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Vascular and glial alterations during aging in wild-type mice and along Alzheimer's disease progression in APP/PS1 mice

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Vascular and glial alterations during aging in wild-type mice and along Alzheimer's disease progression in APP/PS1 mice

Alterações vasculares e gliais associadas ao envelhecimento e à doença de Alzheimer no ratinho APP/PS1

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Abbreviations

Aβ	Amyloid-β
AD	Alzheimer's disease
APP	Amyloid-β precursor protein
APOE	Apolipoprotein E
APOE4	Apolipoprotein E ε4 allele
BACE1	β-secretase 1
BBB	Blood-brain barrier
BM	Basement membrane
CAA	Cerebral amyloid angiopathy
CBF	Cerebral blood flow
CD31	Cluster of differentiation 31
CNS	Central nervous system
CVD	Cerebrovascular diseases
GFAP	Glial fibrillary acidic protein
HIF-1α	Hypoxia inducible factor 1α
Iba-1	Ionized calcium-binding adapter molecule
IL	Interleukin
LRP-1	Lipoprotein receptor-related protein 1
MEOX2	Mesenchyme homeobox-2
MCI	Mild cognitive impairment
MMP	Metalloproteinases
PDGFR-β	Platelet-derived growth receptor β
Pgp	P-glycoprotein
RAGE	Receptor for advanced glycation
sLRP-1	Soluble lipoprotein receptor-related protein
TJ	Tight junctions
USA	United States of America
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WT	Wild-type

Abstract

The blood-brain barrier (BBB) is more than a loyal protecting wall of the central nervous system (CNS). The BBB is a dynamic bidirectional interface between the CNS and blood, formed by endothelial cells, basement membrane, pericytes and astrocytes endfeet. Since its unique properties and location, it is a central player in the maintenance of CNS microenvironment. The communication between the BBB and the neurovascular unit components, microglia and neurons, was found to be crucial for the CNS homeostasis, as it was found to be dysfunctional in aged brain and in Alzheimer's disease (AD) patients brain. Based on this, we aimed to investigate which vascular and glial events are characteristic of AD or/and aging, as well as to establish the temporal evolution of these changes in AD-like APP/PS1 and wild-type (WT) mice. Moreover, we aimed to relate these changes with amyloid-β (Aβ) accumulation. We used hippocampi and cortex to analyze the temporal evolution of selected parameters in a young adult, a middle age and an old age group. Our results show that aging is the main factor contributing to the upregulation of receptor for advanced glycation endproducts and desmin, as well as to the entrance of thrombin and albumin in hippocampus parenchyma. On the other hand, AD was found to be the unique contributing factor to the loss of platelet-derived growth factor receptor-β (PDGFR-β) positive cells, in both studied regions. Both factors contributed to hypovascularization in hippocampus, but in cortex it was just a reflex of the interaction between both factors. Astrogliosis was a result of AD in hippocampus and it is a reflex of both factors in cortex, while microgliosis is a result of AD and the interaction between both factors in both regions. Regarding the relationship between glia-vascular changes and senile plaques, we found that senile plaques precede vascular and glial alterations in hippocampus. Interestingly, in cortex, vascular and glial alterations, specifically loss of PDGFR-β-positive cells and astrogliosis, accompanied the first senile plaques. In sum, this study points to vascular and glial events that can underline AD pathogenesis and age-related brain vulnerabilities.

Key words: Endothelial cells, pericytes, blood-brain barrier disruption, glial activation, Alzheimer's disease, aging.

Resumo

A barreira hematoencefálica (BHE) é mais do que uma simples barreira protetora do sistema nervoso central (SNC). A BHE é uma barreira dinâmica e bidirecional entre o SNC e o sangue, formado por células endoteliais, membrana basal, pericitos e as terminações dos astrócitos. O facto de a BHE estar localizada numa posição privilegiada e de ter propriedades únicas, permite-a desempenhar funções de manutenção na homeostasia do SNC. A perturbação da comunicação entre a BHE e os elementos da unidade neurovascular, a microglia e os neurónios, parece ser uma característica do envelhecimento e da doença de Alzheimer (DA). Deste modo, o objetivo deste trabalho foi investigar se as alterações vasculares e gliais são características do envelhecimento e/ou da DA e estabelecer a evolução temporal dessas alterações ao longo do envelhecimento, em ratinhos saudáveis (*wild-type*), e da progressão da doença, utilizando o modelo APP/PS1 que mimetiza a DA. Além disso, essas alterações foram relacionadas com a densidade das placas senis. Foram utilizados o hipocampo e o córtex de três grupos diferentes, um grupo de jovens adultos, um grupo de indivíduos de meia-idade e um terceiro grupo constituído por indivíduos idosos, de modo a analisar a evolução temporal dos parâmetros selecionados. Os resultados obtidos demonstram que o envelhecimento é o principal fator que contribui para o aumento da expressão do recetor dos produtos avançados da glicação e de desmina, bem como para a entrada de trombina e albumina para o parênquima do hipocampo. Por outro lado, a perda de células positivas para o recetor do fator de crescimento derivado de plaquetas (PDGFR-β) em ambas as regiões foi o resultado da DA. Ambos os fatores estudados contribuíram para a hipovascularização no hipocampo, mas no córtex foi um resultado da interação entre ambos os fatores. A astrogliose é o resultado da DA no hipocampo, enquanto que no córtex isso é o resultado de ambos os fatores. A microgliose é afetada pela DA e pela interação entre ambos os fatores em ambas as regiões. Considerando a relação entre as alterações gliais e vasculares com o aparecimento de placas senis, foi estabelecido que as placas senis precedem as mudanças gliais e vasculares apenas no hipocampo. Interessantemente, no córtex as relações gliais e vasculares, nomeadamente a perda de células positivas para o PDGFR-β e a astrogliose, são acompanhadas pelo aparecimento de placas senis. Deste modo, este estudo aponta para o facto de as alterações vasculares e gliais podem estar associadas à patogénese da DA e à vulnerabilidade do cérebro a patologias associadas à idade.

Key words: Células endoteliais, pericitos, disrupção da barreira hematoencefálica, ativação glial, doença de Alzheimer, envelhecimento.

Chapter I

Dissecting the contribution of vascular alterations and aging to Alzheimer's disease

The work presented in this chapter originated the following manuscript:

Dissecting the contribution of vascular alterations and aging to Alzheimer's disease
Janota C, Lemere CA, Brito MA
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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease characterized by cognitive decline that afflicts as many as 45% of individuals who survive past the age of 85. AD has been associated with neurovascular dysfunction and brain accumulation of amyloid-β (Aβ) peptide, as well as tau phosphorylation and neurodegeneration, but the pathogenesis of the disease is still somewhat unclear. According to the amyloid hypothesis for the pathogenesis of AD, accumulation of Aβ peptide aggregates initiates a cascade of events leading to neuronal injury and loss and, eventually, dementia. Alternatively, the vascular hypothesis of AD incorporates the vascular contribution to this disease cascade, stating that a primary insult to brain microcirculation (e.g., stroke) contributes not only to amyloidopathy, but initiates a non-amyloidogenic pathway of vascular-mediated neuronal dysfunction and injury, which involves blood-brain barrier dysfunction, with increased permeability of blood vessels, leakage of blood-borne components into the brain and, consequently, neurotoxicity. Vascular dysfunction also includes a diminished brain capillary flow, causing multiple focal ischaemic or hypoxic microinjuries, diminished Aβ clearance, and formation of neurotoxic oligomers, which lead to neuronal dysfunction. Here, we present and discuss relevant findings on the contribution of vascular alterations during ageing to AD, essential to better understand the players in the orchestra of neurodegeneration and to develop strategies to modulate the symphony.

Key-words: Aging, Alzheimer's disease; blood-brain barrier; glial activation; neurodegeneration; vascular dysfunction.

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Introduction

In a world where the population is aging, the age-related diseases, such as dementias, constitute one of the most feared diseases. Alzheimer's disease (AD) is the most common form of dementia. AD is the sixth leading cause of death and the fifth for those aged 65 and older in the United States of America (USA) (Thies et al., 2013). It is estimated that in 2013, AD cost to USA was $203 billion and, strikingly, this number is expected to rise to $1.2 trillion by 2050 (Thies et al., 2013). It is estimated that AD affects 26 million individuals worldwide, however, that number is projected to rise to nearly 34 million by 2025 and to triple by 2050 (Hebert et al., 2001; Protsenko and DeGiorgis, 2014). Even more concerning, there was an increase of 68% in the number of AD-related deaths between 2000 and 2010 in the USA (Thies et al., 2013). Over the past several years, a number of studies have suggested that in AD there is a very special relationship between neuronal, glial and vascular events. As a result, today there is a very large interest to better understand the neurovascular alterations that occur in AD and to determine whether they lead to or result from Aβ accumulation. Thus, in this review we will focus not only on the alterations that are observed in the brain parenchyma in AD, but also on neurovascular events that occur with normal aging and may predispose the elderly to AD.

1. Alzheimer's disease

1.1 Pathogenesis of Alzheimer's disease

AD is a progressive, irreversible and neurodegenerative pathological disease. There are two main types of AD; as many as 95% of AD cases are sporadic and have a late onset, whereas about 5% of cases are familial and present an early onset (Hunter et al., 2013). Whereas the familial form of AD is due to mutations in three main deterministic genes coding for Aβ precursor protein (APP), presenilin 1 and 2, the sporadic form of AD seems to be related to several risk genes and environmental factors, such as apolipoprotein E ε4 allele (APOE4) and stress (Piaceri et al., 2013).

Clinically, AD usually manifests with cognitive impairment, memory loss, disorientation, as well as altered mood and behavior (Kumar et al., 2012). Neuropathologically, AD is characterized by intracellular neurofibrillary tangles of hyperphosphorylated tau (tauopathy), Aβ extracellular aggregates (amyloidopathy), inflammation, oxidative stress and premature neuronal apoptosis (Serrano-Pozo et al., 2011). Three main events are thought to ause tauopathy: the first is the imbalance between hyperactivated kinases and hypoactivated phosphatases that results in hyperphosphorylated isoforms of tau protein; the second is tau truncation, as a result of the activity of different proteases; and finally, the third is the interaction of tau with sulphated glycosaminoglycans (Goedert et al., 2006; Hanger et al., 2009;

Kovacech and Novak, 2010; Wang et al., 2010a). After decades of research, AD is widely believed to be driven by early accumulation of Aβ aggregates, including oligomers, in the parenchyma and in the blood vessels, a condition known as cerebral amyloid angiopathy (CAA), followed by tau hyperphosphorylation and aggregation, and finally neurodegeneration.

Aβ pathological accumulation is a result of the increased proteolytic cleavage of APP by β- and γ-secretase, whose major component is presenilin, and/or reduced clearance. The amyloidopathy is characterized by microvascular dysfunction, and consequently, changes in intracellular pH due to alterations in the transport of water and electrolytes (Zlokovic, 2008a). These variations are attributed to a loss of activity of several energy-dependent ion pumps, such as sodium/hydrogen exchanger and ATP-dependent sodium pump (Zlokovic, 2008a). All of these events contribute to the degeneration of the brain, shown by neurovascular disintegration (Brown and Thore, 2011; Kalaria, 2010).

More than 200 genes are related to AD and most of them are linked to mutations that affect the processing, trafficking and recycling of Aβ (McDonald et al., 2010). A recent study identified 11 new susceptibility *loci* related to late onset AD, including the HLA-DRB5-HLA-DRB1 that is related with immunocompetence and histocompatibility (European Alzheimer's Disease et al., 2013). This is very interesting because it strongly reinforces a possible involvement of the immune system in AD and, additionally, this region is also related with multiple sclerosis and to Parkinson's disease, both of which are thought to have a major immune components (International Parkinson Disease Genomics et al., 2011; Sawcer, 2011). The second strongest association was within the sortilin-related receptor 1 gene, which is also of interest because it is associated with increased risk of both familial and sporadic forms of AD (European Alzheimer's Disease et al., 2013). In addition, sortilin-related receptor 1 is the first late onset AD gene that directly links abnormal trafficking and metabolism of APP to sporadic AD (Pottier et al., 2012; Rogaeva et al., 2007). A major contributing gene to sporadic late-onset AD is APOE, and as many as 65-80% of all AD patients are carriers of the APOE ε4 allele (APOE4) (Rohn, 2013). Recently, it was found that the APOE ε4 phenotype begins even before birth, since APOE4 heterozygous neonates, compared to APOE ε3 homozygotes, presented diminished gray matter volume in the temporal lobes, including hippocampus, and simultaneously larger parietal lobe volume (Knickmeyer et al., 2014). This study contributed a new perspective about AD, since almost all previous studies examined adult brain. Given the importance of the APOE ε4 genotype, it has become a potential therapeutical target for AD. For example, AD transgenic mice treated with bexarotene, an inducer of APOE transcription, showed a more than 50% reduction in Aβ burden just 72 hours after administration (Cramer et al., 2012).

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1.1.1 The old and new Alzheimer's disease paradigms

Over the last twenty years, the most popular AD theory was the amyloid hypothesis, represented in figure 1. However, the role of Aβ in the onset and progression of AD is not fully known and the amyloid hypothesis continues to be discussed and modified. The original amyloid hypothesis states that Aβ initiates a cascade of events leading to neuronal injury and loss (Hardy and Selkoe, 2002), and cognitive impairment (Cummings, 2004). However, the underlying mechanism through which soluble Aβ aggregates or Aβ plaques exert neurotoxicity responsible for synaptic impairment and cognitive decline in early AD are still unclear. A study using Tg2576 AD mice model found that blood-brain barrier (BBB) dysfunction precede senile plaques deposition, as well as cognitive impairment (Ujiie et al., 2003). This suggests that further studies are needed to understand the role of early soluble Aβ aggregates as well as other non-Aβ mechanisms that promote central nervous system (CNS) dysfunction. Moreover, it is also important to mention that at least some of the contradictory data about AD mice overexpressing APP could be related to differences in familial mutations and promoters, as well as variability in protein expression levels. Thus, despite having the similar phenotypes, it is very likely that the same therapeutic approach may not benefit all AD patients.

Since 2005, several authors have been suggesting a hypothesis to AD based on a two-hit theory, known as vascular hypothesis. Several epidemiological, pathological, neuroimaging, pharmacotherapeutic and clinical studies support that vascular dysfunction is an integral part of AD pathogenesis (de la Torre, 2010a; Marchesi, 2011; Zlokovic, 2005). While the amyloid hypothesis states that neuronal loss (Hardy and Selkoe, 2002) and cognitive decline (Cummings, 2004) are a result of Aβ cascade, the vascular hypothesis goes further and states that vascular risk factors, as well as aging, contribute to vascular dysfunction (hit 1), which contributes to Aβ accumulation in the parenchyma and in the blood vessels (hit 2), as depicted in figure 1. The first hit is characterized by reduced cerebral blood flow (CBF), hypoxia and BBB dysfunction, which decreases clearance of potentially vasculotoxic and neurotoxic molecules from the parenchyma (de la Torre, 2010a, b; Marchesi, 2011). Some studies supporting the vascular hypothesis show that the cerebrovascular dysfunction may lead to faulty Aβ clearance from the brain (Deane et al., 2004a; Deane et al., 2004b), augmented influx of peripheral Aβ through the BBB (Eisele et al., 2010) and overexpression of APP (Kumar-Singh et al., 2005; Weller et al., 2008). Altogether, these events contribute to Aβ accumulation both in the parenchyma and blood vessels. The vascular hypothesis overlaps with the amyloid hypothesis insofar as both support the idea that augmented levels of Aβ in the cerebral parenchyma accelerate neurovascular (Bell and Zlokovic, 2009) and neuronal damage (Takuma et al., 2009). In addition, recent studies also show that both brain and circulating Aβ1-40 are not a prerequisite for AD vascular dysfunction, however CAA still contributes

to functional deficits (Park et al., 2013). More studies are required to understand, for example, the temporal relationship between vascular damage and glial activation.

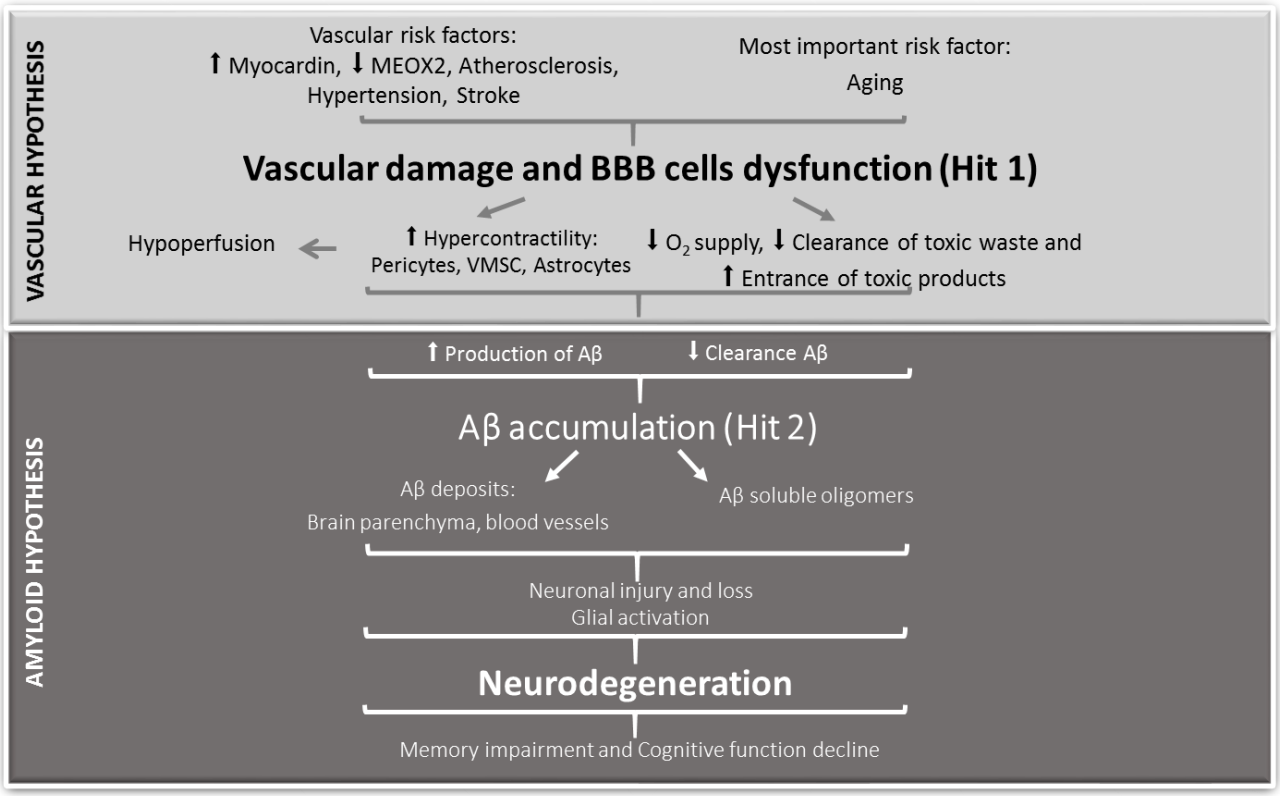

Fig. 1. Schematic representation of the amyloid and the vascular theories for Alzheimer's disease (AD) pathogenesis. According to the amyloid theory, an increased accumulation of amyloid-β (Aβ) peptide, together with its reduced elimination, leads to the formation of soluble Aβ oligomers and Aβ deposition in brain parenchyma, triggering a cascade of events that result in neurodegeneration and cognitive impairment. The vascular hypothesis states that vascular risk factors and aging contribute to vascular damage and blood-brain barrier (BBB) dysfunction (hit 1), which lead to hypoperfusion and to the entrance in brain parenchyma of blood-borne molecules and ensuing neurotoxicity, respectively. These vascular events drive Aβ accumulation in the parenchyma and in the blood vessels (hit 2) that, in turn, potentiate to neurodegeneration.

1.1.2 Evidences of a link between cerebrovascular diseases and Alzheimer's disease

Due to the close proximity and interplay between microvascular endothelial cells and parenchymal cells, changes in microvasculature functioning affect both neuronal and glial activity. The relation between cerebrovascular diseases (CVD) and AD, both sharing several risk factors, has been studied. Epidemiological studies have demonstrated a relationship between CVD and AD, which heretofore had been poorly explored (de la Torre, 2010b; Jellinger, 2010). Hypertension (Iadecola and Davisson, 2008), obesity (Whitmer et al., 2008) and atherosclerosis (Kovacic et al., 2012) are risk factors for both CVD and AD. It is also known that most AD cases have both vascular pathology and small-

vessel disease (Jellinger, 2010; Marchesi, 2011). Other clinical changes can increase the risk of developing AD, such as reduced brain blood perfusion (Ruitenberg et al., 2005) or silent infarcts (Vermeer et al., 2003). In summary, these studies show a wide range of cerebrovascular lesions that might accelerate the onset or the progression of a non-vascular dementia, AD.

CBF regulation plays a central role in AD, because CBF reduction can induce and/or intensify neuropathological changes similar to AD. In animals, CBF changes have large effects on Aβ and tau signaling pathways. The relationship between ischemic insults and amyloidogenesis is supported by one study demonstrating that the activity of β-secretase 1 (BACE1) is increased during ischemic cerebral hemisphere of rodents (Wen et al., 2004). Another recent study indicated that young subjects are more likely to present a higher association between CVD and AD, showing that, even though AD is not a risk factor for strokes or ischemic strokes, AD subjects, particularly younger patients, presented a higher risk of hemorrhagic strokes (Tolppanen et al., 2013). In addition, it was recently showed that vascular risk factors enhance the conversion from mild cognitive impairment (MCI) to AD (Li et al., 2011). Along with these results, the authors found that the treatment of vascular risk factors reduced the risk of AD, suggesting that risk factors may trigger pathological pathways involved in AD. In sum, it is important to realize that several vascular diseases may share a common endpoint with AD, such as brain microvascular damage and/or neurodegeneration, as well as tauopathy and amyloidopathy (Zlokovic, 2011). Although it remains unclear whether neurodegeneration is a primary event or is secondary to vascular impairment, the available data suggest that AD patients should be pharmacologically targeted for CVD along with AD treatment.

1.2 Vascular dysfunction in Alzheimer's disease pathogenesis

BBB breakdown, hypoperfusion, cerebral autoregulation and vascular reactivity, as well as metabolic dysfunction, seem to be highly relevant to AD. Because it remains unknown if the major role and peak of vascular dysfunction happens during AD onset or disease progression, we will present and discuss the main vascular events in AD at cellular and molecular levels.

1.2.1 Blood-brain barrier breakdown in Alzheimer's disease

The BBB is a dynamic interface between the circulating blood and the parenchyma. The endothelium of brain microvasculature forms the anatomic basis of the BBB. Endothelial cells are connected by a junctional complex mostly composed by tight junctions (TJ) that confer low paracellular permeability and electrical resistance to the BBB, and adherens junctions that link adjacent cells by giving place to the adhesion belt. To overcome the restricted paracellular permeability and assure the

entrance into the brain of nutrients, such as glucose, and the exit of molecules, such as Aβ, endothelial cells are equipped with several transporters. Endothelial cells are supported by a basement membrane (BM) and enclosed by pericytes and astrocytes endfeet, which establish important interactions with neurons and microglia within the neurovascular unit (Cardoso et al., 2010; Sá-Pereira, 2012).

Some studies revealed changes in the BBB integrity in AD, however, these alterations are not observed in all AD animal models (Erickson and Banks, 2013). The main factors that contribute to the BBB breakdown in AD are: decrease of pericytes, focal necrosis of the cerebral endothelium, activation of endothelial cells, decreased endothelial mitochondrial density, increased pinocytotic vesicles, increased BM thickness, and reduced TJ proteins as a result of the increased activity of metalloproteinases (MMP) (Sagare et al., 2012). Moreover, other events also contribute to BBB breakdown and to the increase of BBB permeability, e.g. the presence of the blood-borne components into brain parenchyma, such as thrombin, which induces the activation of astrocytes and microglia. Figure 2 presents the main factors that contribute to BBB disruption, compared to what happens in the functional BBB of healthy individuals.

Although little is known about the molecular and cellular mechanisms underlying the loss of BBB integrity and whether the soluble or insoluble Aβ forms are responsible in any way, *in vitro* studies showed that Aβ influences the expression and localization of TJ proteins (Tai et al., 2010) and that low levels of several TJ and AJ proteins have been demonstrated in AD (Kalaria, 2010; Zlokovic, 2008a), which explains the impairment of BBB properties. Moreover, the expression of messenger RNA encoding essential TJ proteins is decreased in AD (Henkel et al., 2009). Furthermore, the TJ proteins and BM extracellular matrix proteins are substrates of the vascular-associated MMP, whose activity is also increased in AD and after ischemic CNS injury (Rosenberg, 2009).

Recent evidence points to pericytes as key players in the impairment of BBB properties in AD, raising the hypothesis that Aβ affects pericyte functioning by still poorly known molecular mechanisms. Pericytes are not only essential for BBB physical integrity, but their communication with endothelial cells also plays a key role in the formation and maintenance of the BBB. Pericytes promote endothelial TJ protein expression (Bell et al., 2010), help TJ alignment (Daneman et al., 2010) and decrease vesicular uptake and endothelial transcytosis of macromolecules present in the blood (Armulik et al., 2010). Pericytes also concentrate and degrade several circulating exogenous (Broadwell and Salcman, 1981) and endogenous proteins like serum immunoglobulins, fibrin, thrombin and plasmin (Bell et al., 2010), which are not supposed to pass through the BBB. Recent studies by Zlokovic and co-workers (Sagare et al., 2013b; Sengillo et al., 2013) demonstrated that a loss of pericytes is accompanied by a decreased expression of TJ proteins, microvascular hyperpermeability, and leakage into brain parenchyma of blood-

borne molecules, which are noxious to the brain. Moreover, Bell and colleagues showed in an AD mouse model overexpressing APP crossed with platelet derived growth factor receptor (PDGFR)-β-deficient mice that pericyte loss elevated brain $A\beta_{1-40}$ and $A\beta_{1-42}$ levels and accelerated amyloid angiopathy and cerebral β-amyloidosis, since it decreased $A\beta_{1-40}$ and $A\beta_{1-42}$ clearance from brain interstitial fluid resulting in Aβ accumulation (Bell et al., 2010; Sagare et al., 2013b). However, since the animal model used in this study has a prior mutation on PDGFR-β coding gene, it is not possible to confirm if pericyte-mediated vascular damage could an upstream event in AD pathogenic cascade. Better animal models of the disease are required to better understand the role of pericytes within the development of sporadic AD, since the most studies are performed using mouse models with previously identified familial AD mutations; however, this does not mean that patients with familial AD will not benefit from pericyte-focused therapies.

Pericyte loss seems to not only affect amyloidopathy, but also to promote the development of tau pathology and contribute to early neuronal loss, triggering cognitive decline (Sagare et al., 2013b). Because pericytes are in direct contact with Aβ fibrils in the brain, they contribute to its clearance (Wisniewski et al., 1992), and in addition, pericyte loss presumably impairs this function of perivascular cells. On the other hand, high concentrations of $A\beta_{1-42}$ and $A\beta_{1-40}$ may induce human pericytes degeneration (Verbeek et al., 1997), supported by the observed depletion of pericytes in the brain of AD patients (Sengillo et al., 2013). Therapeutics targeting pericytes seem to be a very interesting alternative from the failure of Aβ-lowering drugs to date. However, it is important to consider the consequences of using pericyte proliferation or migration stimulating factors, like PDGFRs and PDGFs, which are expressed by pericytes and endothelial cells, respectively. Indeed, those molecules play critical roles in mesenchymal cell migration and proliferation and autocrine growth stimulation of tumor cells, regulating tumor stroma fibroblast function and tumor angiogenesis (Kim et al., 2012; Mancuso et al., 2006), so that increased expression of PDGFRs and/or PDGFs would provide a favorable microenvironment for the growth and survival of cancer cells. Apoliprotein E (APOE) is an apolipoprotein thought to play a role in the generation of parenchymal amyloid extracellular plaques as well as Aβ transport within CNS. In 2012 it was found that one reason why APOE4 carriers have higher probabilities of developing AD is because they present neurovascular dysfunction, which is associated with the activation of the pro-inflammatory cyclophylin A–nuclear factor-κB–matrix-metalloproteinase-9 pathway in pericytes (Bell et al., 2012). Recently, it was reported that transgenic mice expressing APOE4 isoforms present dysregulation in the expression of Pin1, sirtuin 1 and PS1 genes in different cerebral areas, explaining at another molecular level the higher vulnerability of APOE4 carriers to the disease (Lattanzio et al., 2014).

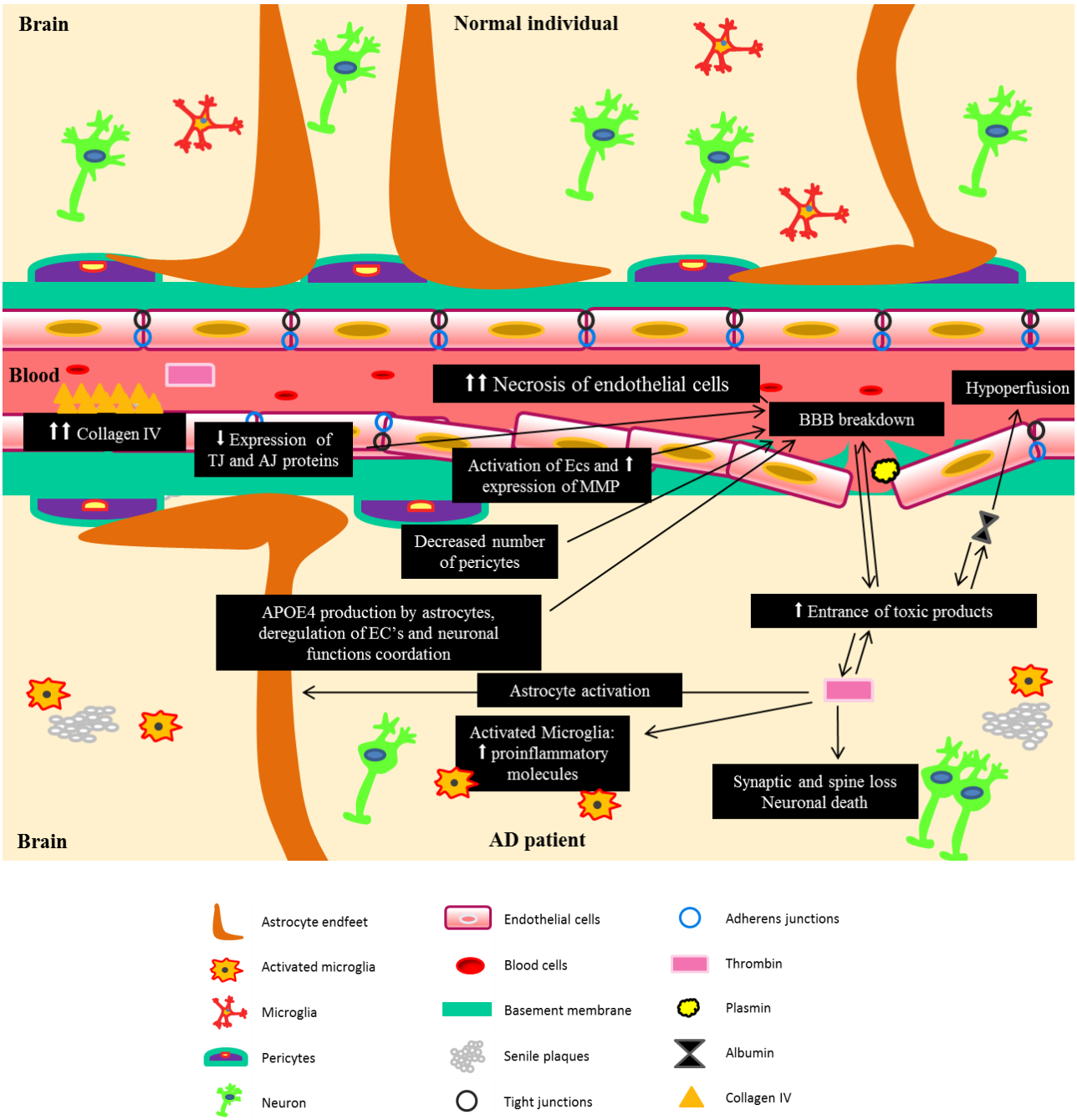

Fig. 2. Schematic representation of the main factors that influence the blood-brain barrier (BBB) breakdown in Alzheimer's disease (AD), compared to what happens in a healthy brain. In a healthy brain there is an equilibrium in neurovascular unit, which is mandatory for the maintenance of BBB integrity. In AD patients' brain, there is a decrease of pericytes, focal necrosis of the cerebral endothelium, activation of endothelial cells (EC) and release of metalloproteinases (MMP) by EC, an increase in basement membrane (BM) thickness, and a decrease of tight junction (TJ) proteins as a result of the increased activity of metalloproteinase (MMP). Altogether, these events contribute to BBB breakdown and an increase in BBB permeability, which allows the entrance of blood-borne components into the brain parenchyma, such as thrombin, which induces the activation of astrocytes and microglia and promotes neuronal death.

1.2.2 Cerebral blood perfusion, cerebral autoregulation and vascular reactivity in Alzheimer's disease

Neurovascular coupling needs efficiently innervated pial and intracerebral arteries and an effective response of pericytes and vascular smooth muscle cells (VSMC) to CNS metabolic needs, due to their contractile functions (Bell et al., 2010). In humans, AD is related to extensive CBF reduction, especially in the areas of the frontal and parietal cortex (Johnson et al., 2005; Schuff et al., 2009). However, the relationship between CBF decrease and regional atrophy is not consistent, considering that no correlation between the two events was reported by Schuff and colleagues (Schuff et al., 2009), while an association between clinical, psychometric, hippocampal volume and hemodynamic data was described by Roher et al. (Roher et al., 2012). Along with this, a recent study reported a significantly lower regional CBF in both the bilateral frontal and temporal lobes in AD, whereas it was lower in left frontal and temporal white matter in the vascular dementia group (Gao et al., 2013). It was found that brain vascular volume is reduced already at 6 months of age in triple transgenic AD mice model, even before the appearance of pathological lesions (Do et al., 2014). Contributing to the theory that vascular dysfunction is not related with Aβ burden, a recent study developed a new methodology to measure cerebral blood volume and showed that there was no difference between an AD mouse model and age-matched control mice (Zerbi et al., 2013). Instead, this study suggests that the cause for cerebral blood volume reduction is impaired vasoactivity of capillaries. Furthermore, cerebral vascular reactivity was impaired more severely in bilateral frontal cortices in AD, making easier to distinguish two pathologies that present deep vascular alterations. Another study analyzed the relationship between CBF and subjects' clinical severity and concluded that major hypoperfused areas are the areas that exhibit the most neuropathological alterations in AD (Hu et al., 2010). This corroborates the theory that CBF is associated with brain atrophy and goes along with the notion that less CBF means less nutrients and oxygen in the parenchyma, which would affect CNS cell survival. In 2010, a study demonstrated that even a transient change in CBF can potentiate tauopathy and augment the levels of Aβ for several weeks (Koike MA, 2010). However, a contradictory theory defends that Aβ triggers extensive neoangiogenesis and hypervascularization, which results in increased BBB permeability (Biron et al., 2011). In addition, immunization of Tg2576 AD mice with Aβ peptides neutralized the amyloid, which stopped neoangiogenesis and prevented BBB hypervascularity (Biron et al., 2013). Altogether, this suggests that different levels of Aβ burden may trigger different signaling pathways. Therefore, different stages of AD pathogenesis may result in different vascular density patterns. This implies that different stages of the disease may require different therapeutical approaches.

Several genetic factors that seem to influence CNS blood perfusion in AD patients were found. During the prodromal phase of AD, individuals carrying a mutation in the PS1 gene have a diminished perfusion in the posterior cingulated-cortex and in the hippocampal-amygdaloid complex (Johnson et al., 2001). Equally, APOE4 increases the risk of developing AD because it is associated with a prolonged CBF failure, preceding the onset of AD (Thambisetty et al., 2010). The genomic profiles of endothelial cells of the BBB shows tremendously decreased levels of vascular-restricted mesenchyme homeobox-2 gene (MEOX2) in AD patients (Wu et al., 2005), suggesting that hypoxia may be upstream of the reduction in MEOX2. Decreased expression of MEOX2 leads to proteasome degradation of lipoprotein receptor-related protein (LRP)-1, a very important exit gate to Aβ clearance across the BBB (Zlokovic, 2008a). These findings contribute to explain the aberrant angiogenesis and the premature pruning of capillaries in AD patients, which could result in decreased microcirculation, hypoperfusion and hypoxia. The same study revealed that hypoxia suppresses MEOX2 expression, and concluded that angiogenesis inhibition leads to the accumulation of Aβ, which has anti-angiogenic activity, resulting in a vicious cycle.

Apoptosis and hypoxia are known to increase the stability of BACE1. Caspase-3, one of the most active caspases in AD, increases the expression of an adaptor molecule, the Golgi-localized gamma-ear-containing ARF-binding protein, involved in BACE1 trafficking and stabilization (Tesco et al., 2007). Besides, hypoxia activates the transcription factor hypoxia-inducible factor (HIF)-1α, which can bind to BACE1 promoter and lead to BACE1 gene transcription and increase of the γ-secretase activity (Li et al., 2009). Recent findings suggest that hypoxemia in the prenatal stage of an AD mouse model promoted a more severe phenotype of the disease, characterized by increased phosphorylation of tau, decreased hypoxia-induced factor, and enhanced activation of astrocytes and microglia (Zhang et al., 2013b). Moreover, the expression of myocardin and serum response factor seems to be increased in AD (Chow et al., 2007). These two factors are responsible to promote the VSMC hypercontractile phenotype. Their activity results in hypoperfusion and CAA (Bell et al., 2009). Although the proangiogenic factor vascular endothelial growth factor (VEGF) is augmented in AD (Kalaria et al., 1998), it was also shown that VEGF binds to Aβ and is deposited in plaques. Although VEGF levels are increased, it is less available in its free form in AD than in normal aged brain (Yang et al., 2004). In addition, low serum levels of angionin, an angiogenesis promoter, were reported in AD patients and were found to be related to cognitive decline (Kim and Kim do, 2012). Additionally, an *in vitro* study showed that VEGF significantly prevented Aβ-induced endothelial apoptosis, and in an AD mice model it restored impaired memory behavior by improving vascular survival (Religa et al., 2013). The production of thrombin by endothelial cells in AD seems to be another proteomical dysfunction of these cells. This protein seems

to be an interesting therapeutic target, since thrombin in the brain parenchyma causes inflammation, abnormal angiogenesis and microglia and astrocytes activation (Lee da et al., 2006; Yin et al., 2010). *In vivo*, thrombin is known to induce tau hyperphosphorylation, increase Aβ production and lead to cognitive impairment (Ciallella et al., 1999; Suo et al., 2003). Altogether, these studies validate the multifactorial nature of AD, suggesting that it is very likely that only a therapeutic approach that targets several pathways would be effective.

The role of astrocytes in AD pathogenesis has been studied and it was shown that astrocytes, too, contribute to Aβ clearance by internalization and posterior degradation (Pihlaja et al., 2008). However, the internalization of fibrillar forms of Aβ causes negative changes in astrocytic metabolism, which may be the link between astrocytic dysfunction and AD neuropathophysiology (Allaman et al., 2010). The demonstration of neurovascular uncoupling, including swelling and retraction of astrocyte endfeet, in an early phase of AD with severe CAA observed in an AD animal model reinforced the possibility of such a link (Merlini et al., 2011).

In 2009, a group of investigators discovered that abnormal cerebral autoregulation could be mediated by $A\beta_{1-40}$ (Takeda et al., 2009). Previously, the lack of information regarding the strong relationship between $A\beta_{1-40}$ and abnormal cerebral autoregulation in humans complicated the understanding of AD pathogenesis. It is known that chronic cerebral hypoperfusion and cerebral ischemia increase the APP expression in neurons (van Groen et al., 2005). In figure 3, the main events related to hypoxia and hypoperfusion in AD patients are illustrated, namely the decreased expression of MEOX2 and angiogenesis, as well as increased expression of HIF-1α, and compared to normal vascular homeostasis in healthy brain.

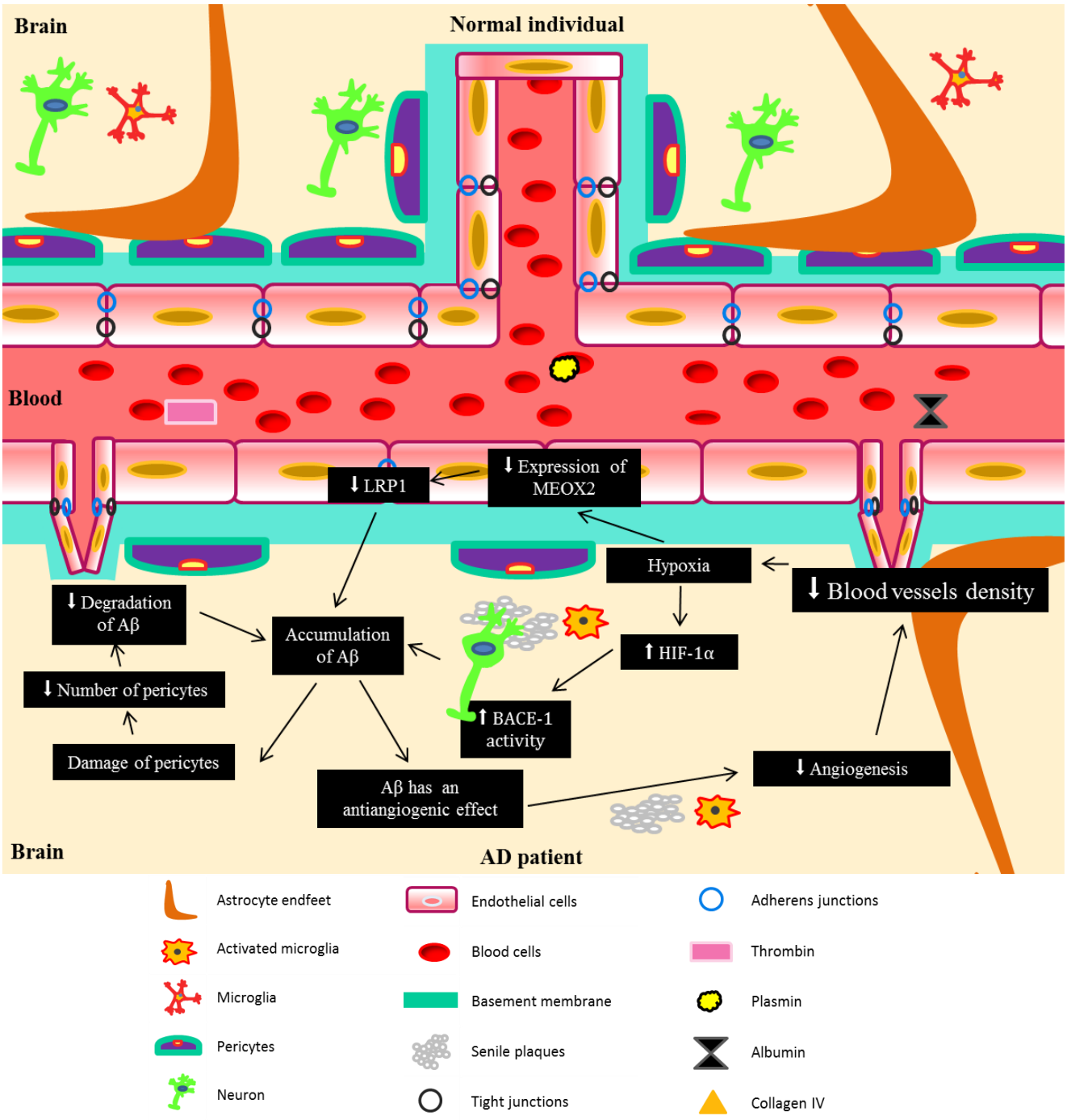

Fig. 3. Schematic representation of the main factors that influence the capillary loss and the cerebral blood flow (CBF) in Alzheimer's disease (AD), compared to normal brain. In AD, there are several events that lead to an intense capillary loss including decreased expression of mesenchyme homeobox 2 (MEOX2) by endothelial cells, which leads to decreased expression of LRP1 by these cells, which along with the increased activity of β-secretase 1 (BACE1) and a decrease in the number of pericytes, contributes to accumulation of amyloid-β (Aβ), which has an anti-angiogenic effect. Altogether, these factors contribute to a decreased density of blood vessels and, furthermore, to hypoxia.

1.2.3 Metabolic changes in Alzheimer's disease

Until recently, it was not believed that metabolism disorders could be the cause of neurodegenerative diseases. However, bioenergetic defects are emerging as possible pathophysiological mechanisms in several diseases (Harris et al., 2012). It is noteworthy to mention that glucose metabolism and cell death regulation are strictly linked and that one of the earliest events in AD is a decrease in cerebral glucose metabolism (Kapogiannis and Mattson, 2011; King and Gottlieb, 2009; Mergenthaler et al., 2012). In a mouse model of AD, decreased expression of glucose transporter 1 was found in both astrocytes and endothelial cells (Merlini et al., 2011), accompanied by impaired transport of glucose and decreased cerebral lactate release during neuronal activation. Also the metabolism of insulin, a protein with unique functions in CNS, has been studied in AD given the CNS insulin resistance that is observed in AD patients. Insulin is almost exclusively produced by the pancreas and it crosses the BBB through an endothelial saturable transporter and affects feeding and cognition through CNS mechanisms. It is noteworthy to mention that these mechanisms are largely independent from glucose utilization. CNS insulin diverges from the peripheral one, since the latter acts mainly as a metabolic regulatory hormone, whereas CNS insulin functions are closer to the actions of the ancestral insulin molecule (Banks et al., 2012). Endothelial cells are responsible for the transport of insulin across the BBB and are themselves regulated by insulin. Factors that alter insulin transport, such as hyperglycemia and the diabetic state, can lead to BBB disruption (Banks et al., 2012). In 2012, it was found that vascular pericytes protect the BBB from diabetes-associated BBB disruption; however, these cells may lose their protective function due to oxidative stress caused by hyperglycemia, suggesting that endothelial cells are susceptible to the diabetic state (Price et al., 2012). Resistance to insulin within the CNS is sometimes referred to as diabetes mellitus type III (Banks et al., 2012). Recently, there has been an intense growth in the literature indicating that insulin deficiency and insulin resistance may be mediators of AD-type neurodegeneration; however, the contribution of type 2 diabetes mellitus, metabolic syndrome and obesity to AD pathogenesis is still being controversial and target of several studies (de la Monte and Wands, 2008).

Metabolic stress, such as cellular starvation, hypoxia and inflammation, can activate autophagy (Kroemer et al., 2010). Some published reports have proposed that the development of neurodegenerative diseases is related to defective autophagy (Harris et al., 2012; Kapogiannis and Mattson, 2011; Kroemer et al., 2010). Recently, a link was described between neurodegeneration and both a disrupted axonal nutrient supply and a defective metabolic network (Funfschilling et al., 2012; Lee et al., 2012). To this end, a constant metabolic equilibrium for neurovascular coupling may be required and may be affected under severe metabolic conditions changes in which the vasodilator reaction of the cerebral vessels may be insufficient (Lin et al., 2011; Wolf et al., 2011). Cerebral hypoperfusion can also contribute to the

decrease of oxygen concentration, which would promote the decoupling of the wave of neuronal activation and the vascular reaction, subsequently resulting in a constriction response (Jespersen and Ostergaard, 2012; Lauritzen et al., 2013). Consequently, these events may lead to cortical spreading depression, endothelial cell activation and disruption of endothelial TJ, which induces BBB lesions and inflammatory response (Stanimirovic and Friedman, 2012).

1.2.3.1 Role of influx and efflux transporters in amyloid-β transport across the blood-brain barrier in Alzheimer's disease

The increased accumulation of Aβ aggregates is one of the earliest and largest hallmarks of AD. Since blood is known to be a major and chronic source of brain Aβ in sporadic AD (Clifford et al., 2007), the accumulation of this protein can result from decreased clearance or increased entrance into CNS parenchyma, especially in sporadic AD (Zlokovic et al., 2000). Therefore, since Aβ's clearance mechanisms are contributing to the disease, targeting the transport systems for Aβ appears to be a possible therapeutic strategy. There are several Aβ-related nonspecific transporters, such as the LRP-1, the low-density lipoprotein-related protein 2 receptor (also known as megalin and glycoprotein 330), the P-glycoprotein (Pgp) and the receptor for advanced glycation endproducts (RAGE) (Deane et al., 2012; Marzolo and Farfan, 2011; Sagare et al., 2012) Whereas LRPs and Pgp are efflux transporters, RAGE is an entrance gate for peripheral Aβ to enter into the brain parenchyma (Zlokovic, 2008a). The main pathways associated with LRP-1 and RAGE are depicted in figure 4.

RAGE is the main receptor responsible for influx of Aβ and it is usually expressed at low levels in the brain, except in the endothelium of brain capillaries and small arterioles (Zlokovic, 2008b). In human brain, RAGE is expressed by neurons, astrocytes, microglia and endothelial cells and is upregulated in the AD brain (Jeynes and Provias, 2008; Park et al., 2004). AD is characterized by the accumulation of RAGE ligands (Semba et al., 2010) and Aβ and RAGE seem to be upregulated in cerebral blood vessels, microglia and neurons (Yan et al., 1996). However, the relationship between RAGE and memory impairment has not been clearly defined. It is not fully understood if RAGE is increased in AD because its ligands are augmented, or if Aβ accumulation in the brain is due to a dysfunctional RAGE upregulation. After RAGE binds to its ligands, it activates an intracellular pathway that results in the production of reactive oxygen species, mitochondrial dysfunction and the activation of transcription of nuclear factor kappa B (Bierhaus et al., 2005; Bierhaus and Nawroth, 2009). Chronic activation of this cascade of events leads to cellular dysfunction (Bierhaus et al., 2005) that affects subsequently affects BBB integrity and the function of neurons, microglia and VSMC, thereby making RAGE a desirable target in AD. Thus, it appears that the interaction between Aβ and RAGE contributes

directly to neuronal killing by promoting oxidative stress damage to RAGE-expressing neurons, and indirectly by activating microglia (Yan et al., 1996). The first molecular mechanism underlying Aβ-RAGE interaction-induced changes in BBB was found recently.

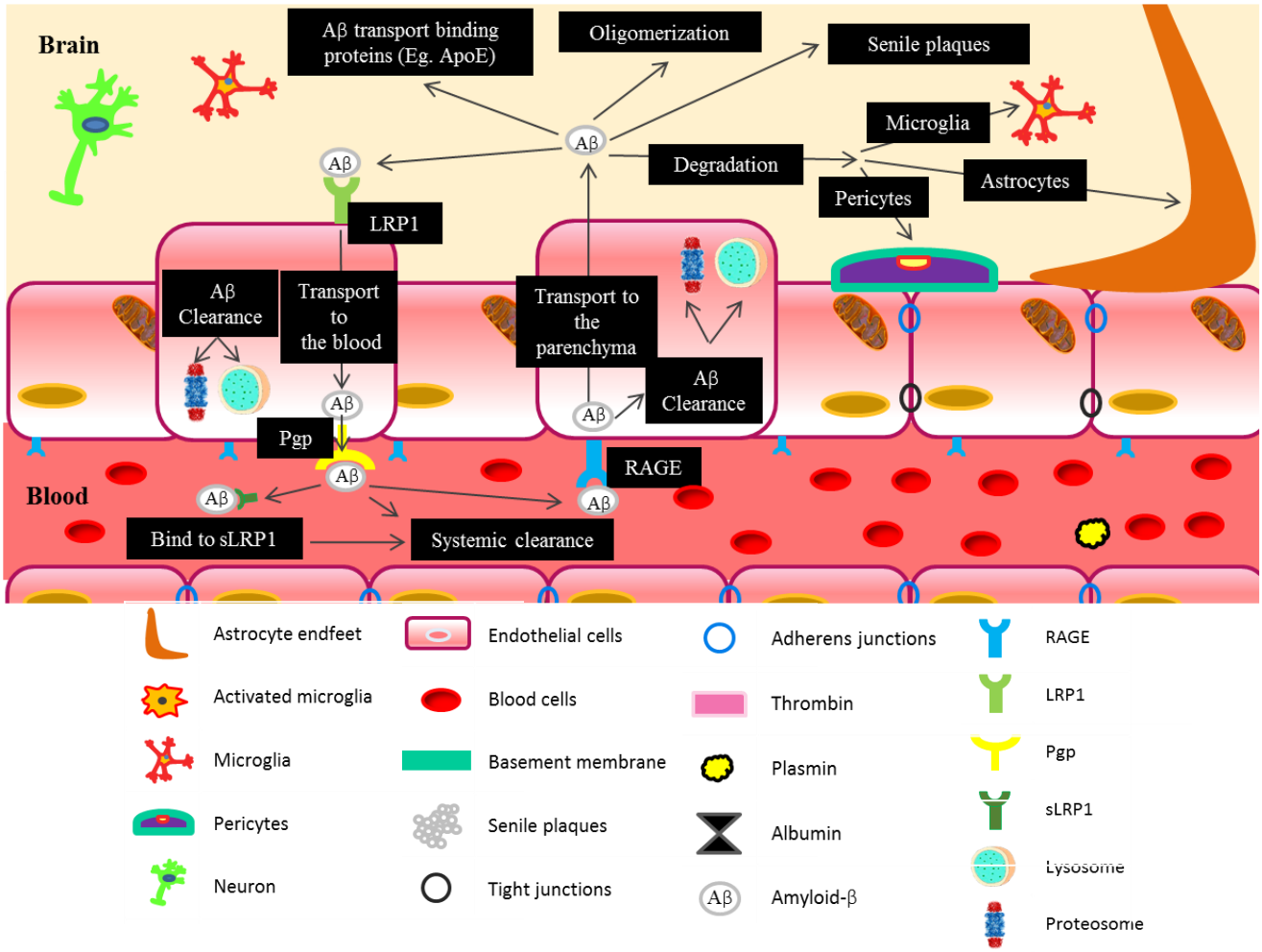

Fig. 4. Schematic representation showing the transport of Aβ across the BBB through the cell surface receptors lipoprotein receptor-related protein 1 (LRP1), receptor for advanced glycation endproducts (RAGE) and P-glycoprotein (Pgp), as well as its interactions with soluble LRP1 (sLRP1) within the blood circulation.

Indeed, Kook et al. (Kook et al., 2012) found that $A\beta_{1-42}$ promotes enhanced permeability, mediated by the disruption of the expression of TJ protein zonula occludens-1 in plasma membrane, and increased intracellular calcium, in addition to MMP secretion in endothelial cells in culture. In the same study, neutralizing antibodies against RAGE and inhibitors of calcineurin and MMP effectively prevented disruption of zonula occludens-1, which led the authors to conclude that Aβ-RAGE interactions influence TJ proteins expression through the Ca^{2+}-calcineurin pathway. In further support of RAGE as a therapeutic target, the high-affinity RAGE-specific inhibitor (FPS-ZM1) prevented Aβ from binding to the V domain of RAGE and also inhibited $A\beta_{1-40}$- and $A\beta_{1-42}$-induced cellular stress in RAGE-

expressing cells not only *in vitro* and also *in vivo* (Deane et al., 2012). In an aged APP transgenic AD mouse model with established Aβ pathology, FPS-ZM1 inhibited RAGE-mediated influx of circulating Aβ40 and Aβ42 into the brain. It resulted in markedly reduced $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in brain and normalized cognitive performance and CBF responses in aged AD transgenic mice. However, its efficacy in humans is unknown. Recent results from a clinical trial show that the use of an inhibitor of RAGE-Aβ interactions was associated with cognitive decline (Galasko et al., 2014). This suggests that RAGE may not be a key factor in AD pathogenesis and that the inhibitor may had deleterious off-targets effects. Despite this finding, TransTech Pharm is scheduled to begin a Phase 3 clinical trial by December 2014. Since the interaction between RAGE and its ligands activates an intracellular pathway that results in reactive oxygen species production, mitochondrial dysfunction, as well as activation of transcription of nuclear factor kappa B (Bierhaus and Nawroth, 2009).

LRP-1 is the major efflux transport for Aβ across the BBB (Shibata et al., 2000) and in human brain it is abundantly expressed by neurons, astrocytic feet processes, VSMC, pericytes and, to a lower extent, by the endothelium. When LRP-1 binds to Aβ at the BBB, it promotes Aβ clearance from brain to blood, through transcytosis (Shibata et al., 2000); furthermore, it internalizes its ligands, resulting in proteolytic degradation in lysosomes or the proteasome. On the other hand, soluble LRP-1 (sLRP-1) is released to plasma, where it binds to 70-90% of plasma Aβ and prevents free Aβ from entering into the brain (Sagare et al., 2007). Reduced LRP-1 expression in the choroid plexus epithelium (Johanson C, 2006) is accompanied by Aβ accumulation in that region (Behl et al., 2009; Behl et al., 2010). LRP-1 downregulation in VSMC leads to Aβ accumulation in the wall of pial and intracerebral arteries and contributes to parenchymal Aβ accumulations (Bell et al., 2009; Kanekiyo et al., 2012). LRP-1 also seems to interact with APP, promoting Aβ production through γ-secretase and inhibiting the inflammatory response (Waldron et al., 2008; Zurhove et al., 2008). LRP-1 is similarly reduced in AD and aging in humans, nonhuman primates, and rodents, as well as in AD models and AD patients (Bading et al., 2002; Deane et al., 2004a). In AD patients and AD animal models, Aβ binding to sLRP-1 is diminished due to oxidation, which increases the levels of free circulating Aβ (Sagare et al., 2007). This seems to create a link between RAGE and LRP-1, because oxidation results in increased uptake of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ by RAGE (Deane et al., 2003; Donahue et al., 2006; Sagare et al., 2007). It is interesting that RAGE dysfunction and upregulation, which could result in increased uptake of Aβ from the blood to the brain and promote oxidative stress, might be related to an increase in circulating Aβ, given that sLRP-1 is oxidized. However, this relationship has not yet been confirmed. Recently, it was reported that complement protein C1q protected both immature and mature primary cortical neurons against fibrillar and oligomeric Aβ-mediated neurotoxicity possibly through a neuroprotective response that involves the

upregulation of LRP1B (Benoit et al., 2013). Further evidence of the neuroprotective role of LRP1 was recently found: LRP1-dependent cell signaling via TrkC activation promoted axonal growth in the CNS after injury, suggesting a clear contribution of LRP-1 to neuronal plasticity (Yoon et al., 2013). Sagare and colleagues (Sagare et al., 2013a) further explored LRP-1 as a target by showing that 3-month treatment of an AD mouse model with LRPIV-D3674G, a mutant receptor with higher affinity to Aβ40 and Aβ42 *in vitro* and lower affinity to other LRP-1 ligands, given subcutaneously at 40 μg/kg/day, reduced $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in the hippocampus, cortex and cerebrospinal fluid by 60-80% and enhanced CBF and hippocampal function at 9 months of age. Hence, LRPIV-D3674G has good potential as an Aβ efflux therapy.

Also, the contribution of Pgp to the CAA development has been discussed over the last few years, as single-nucleotide polymorphisms in the Pgp gene found in AD patients might render Pgp dysfunctional, thereby contributing to the accumulation of intravascular Aβ (van Assema et al., 2012b; van Assema et al., 2012c). Several studies have been trying to understand the mechanisms behind CAA genesis, and the increased efflux of Aβ to the blood vessels wall has been a proposed mechanism. However, a recent study showed that almost all AD patients showed some degree of vascular Aβ and only a minority of them showed by magnetic resonance imaging signs of CAA (e.g., microbleeds) during life. While it is possible that only patients with severe CAA show microbleeds on magnetic resonance imaging, an alternative explanation might be that the severity of CAA is not strongly linked to the presence and number of microbleeds (van Assema et al., 2012a).

2. Aging as a predisposing factor for Alzheimer's disease

The aging process is typically accompanied by a progressive failure of CNS functioning, which is more intense in AD. However, little is known about what separates healthy brain aging from the aging accompanied by MCI or AD. Thus, there is a large interest in understanding the cellular and molecular differences between the processes of non-pathological and pathological brain aging. A recent study suggested that brain volume reduction, which has been used to characterize AD, is also a general feature of normal aging (Fjell et al., 2013). Furthermore, amyloidopathy, CAA and tauopathy are also observed in the brains of 25% of non-demented older adults at autopsy (Schneider et al., 2007). Although not necessarily accompanied by cognitive decline, aging leads to clinically measurable behavioral changes, including decreased processing speed and long-term memory. Also structural changes can be observed, including decreased volume of frontal and parietal regions and changes in white matter integrity (Madden et al., 2012; Salat et al., 2004).

Improving our understanding the neurovascular alterations that predispose elderly to age-related brain vulnerabilities and how these contribute to neurodegenerative disease process should advance the search for new treatments. Furthermore, identifying the characteristics of aging associated specifically with neurological disorders like AD would open new avenues for early diagnosis and timely therapeutic intervention, essential to improving the success of prevention and/or treatment of this devastating and prevalent disease.

2.1 Age-associated vascular alterations

Age strongly contributes to two main vascular events: compromise of BBB properties and alterations of the microvasculature. Mounting data suggest that these changes may be related to age-related cognitive decline, due to the influence of both on neuronal and glial activity. Recent findings showed that aged rats with memory impairment have reduced dopamine levels in the absence of neuronal degeneration; however, these animals also displayed robust vascular and microglial degeneration, associated with decreased VEGF and elevated astrogliosis in the hippocampus (Zhang et al., 2012). Thus, changes within the vascular-neuronal-glial crosstalk could represent major contributing factors to age-related cognitive impairment. Here, we will summarize and discuss the evidence for vascular alterations occurring in the aged brain and how they may contribute to aged-related brain vulnerabilities in the elderly population, and then compare them with vascular alterations in AD brain. The most relevant factors are depicted in figure 5.

2.1.1 Impairment of the blood-brain barrier properties in aging

Age-related changes in BBB permeability include focal necrosis of the endothelium, accumulation of extracellular matrix components in the vascular BM, loss of TJ proteins, changes in astrocyte end-feet and degeneration of the microvascular tree (Kalaria, 1996). Aging also manifests as decreased endothelial mitochondrial density, increased pinocytotic vesicles and stiffening of the blood vessels walls, accompanied by loss of elasticity of vessel walls that affects CNS perfusion, effects that are also observed in AD (Bell and Zlokovic, 2009; Sagare et al., 2012). Moreover, a meta-analysis undertaken by Farral and Wardlaw (Farrall and Wardlaw, 2009) showed an increase of BBB permeability with age. Nevertheless, the authors warned about the significant heterogeneity between studies, which indicates that more data is needed to establish a relationship between aging and BBB disruption. Also, the effects of changes in BBB permeability in AD remain to be clearly established. Recent findings suggest that age-dependent reduced CBF, increased permeability of BBB and reduced expression of glucose transporter 1 and -3 were strongly related to age-related cognitive decline in the SAMP8

senescence mouse model (Zhang et al., 2013a). Interestingly, BBB dysfunction along aging is accompanied by reduced LRP-1 and Pgp (Silverberg et al., 2010a), while RAGE expression increases (Silverberg et al., 2010b).

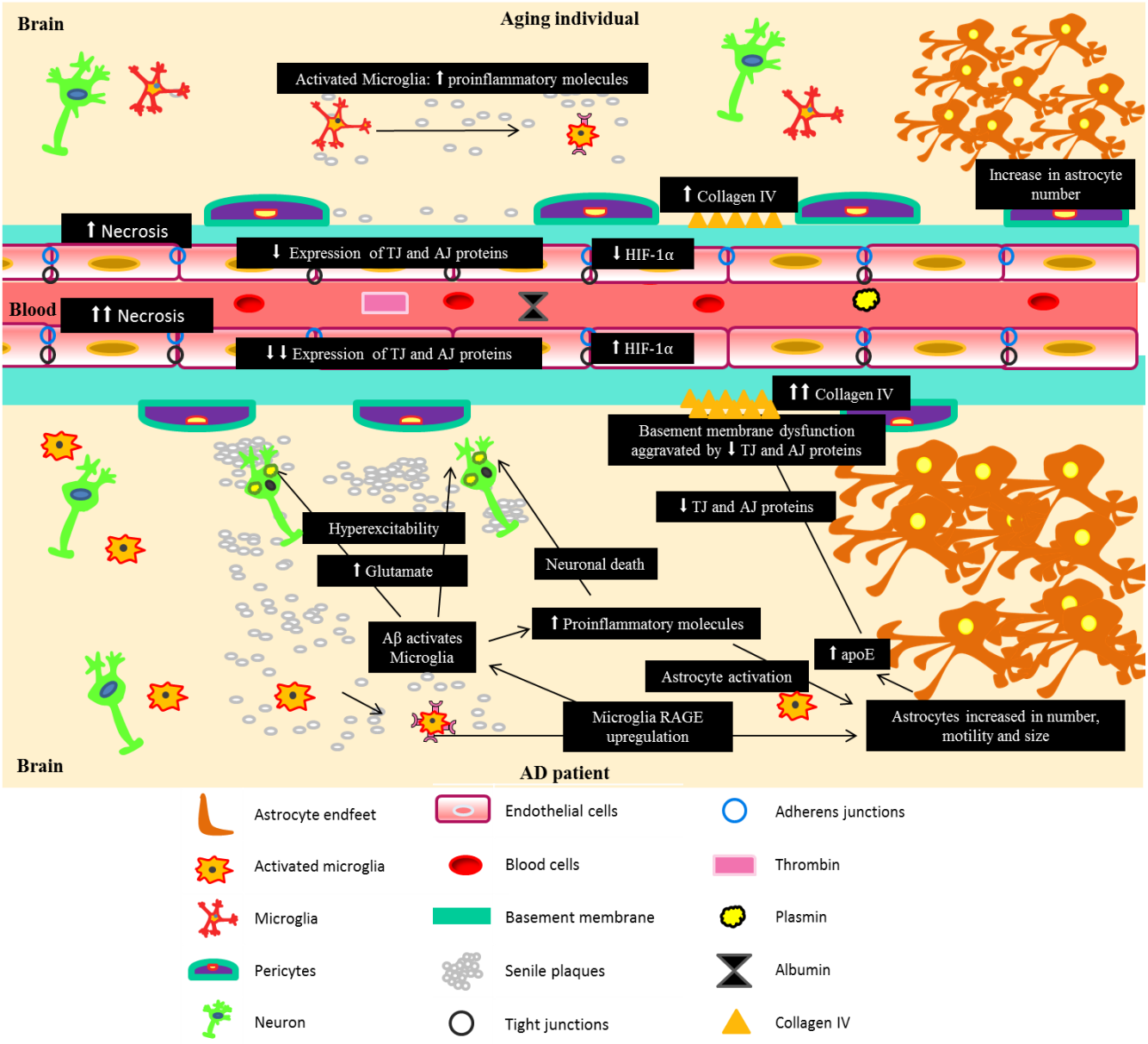

Fig. 5. Schematic representation of the main factors that contribute to glial activation, blood-brain barrier (BBB) disruption and neuronal damage in brain in aging and Alzheimer's disease (AD). While along normal aging the main to glial dysfunction manifests through microglia activation and increased astrocyte number, in Alzheimer's disease (AD), glial activation seems to manifest through multiple events, such as the release of glutamate by microglia that contributes to neuronal hyperexcitability, activation of astrocytes, and astrocytic production of apolipoprotein E (APOE) that leads to the reduction of endothelial tight junction (TJ) proteins, for example. The main vascular events that contribute to BBB disruption along aging are endothelial necrosis, a reduction in TJ proteins, and the accumulation of collagen IV. In AD, these changes are even more robust, which may explain the severe BBB dysfunction observed in AD patients.

BBB disruption is also implicated in other mechanisms that could trigger pathological features. For example, decreased BBB integrity is accompanied by infiltration of T cells and macrophages, which is accompanied by increased expression of chemokines, monocyte chemotactic protein-1 and IFN γ-induced protein-10 (IP-10) (Denieffe et al., 2013). Therefore, given its contribution to age-related brain vulnerabilities, large efforts are underway to restore BBB function. It was found that even though the pituitary adenylate cyclase-activating polypeptide did not increase cerebral microvascular endothelial cell survival, it striking improved transendothelial electrical resistance (Wilhelm et al., 2014). Moreover, it showed that in addition to improving BBB improving BBB tightness, this compound also protected against glucose deprivation and oxidative stress-induced junctional damage. The BM also suffers changes along aging, becoming thicker as age increases (Alba et al., 2004; Farkas et al., 2006). Interestingly, this alteration also occurs, and is even more evident, in AD (Kalaria and Pax, 1995). Age-related BM thickening is a result of the deposition of collagen IV in and around the BM, and is correlated with atherosclerosis in large peripheral vessels (Farkas et al., 2006). However, it remains unclear how the thickening of the BM is involved in BBB impairment. Also intriguing is the association of the thicker BM with the loss of pericytes, considering that these cells contribute to the synthesis of molecular components of the BM. In monkeys, it was reported that the thickness of the outer basal lamina and the increased numbers of its splits, multiple thin electron-dense layers altering with electron-lucent layers, have no relationship with cognitive status (Peters and Sethares, 2012).

2.1.2 Changes in capillary density and cerebral blood flow in aging

Aging is accompanied by a regression in global and regional measures of CBF (~4mL/min/year), cerebral metabolic rate for oxygen, glucose oxidation and cerebral blood volume (Stoquart-ElSankari et al., 2007). Moreover, CAA is related to atrophy in the VSMC layer of small arteries, which may contribute to the more intense capillary loss in AD compared to that seen in normal aging (Cordonnier and van der Flier, 2011). It is known that normal aging also includes a large increase in the vascular tortuosity of arterioles in the deep white matter, and an age-related decrease in capillary number and length (Thore et al., 2007). On the other hand, a previous study showed a decrease in capillary number of 20% in the hippocampus and of 25% in the cortex (Jucker et al., 1990), but little is known about the microvascular density in other brain regions that are strongly affected in AD. Such changes in normal aging may be related to tissue hypoxia that, in a young or adult brain, would initiate an angiogenesis response mediated by HIF-1α, as indicated above. However, the activity of HIF-1α decreases with aging (Wu et al., 2009), which results in an impairment of angiogenesis in the aged brain. This may explain the hypovascularization of the aged-brain that leads to reduced supply of oxygen and

nutrients, while simultaneously decreasing the clearance of molecules such as Aβ. These events contribute to the cascade that leads to neurodegeneration and cognitive impairment, as depicted in figure 1. Furthermore, when young adult mice was exposed to hypoxia and returned to normoxia, the number of blood vessels was not decreased, but when aged brain was exposed to the same situation, this plasticity was markedly reduced possibly due to the absence of hypoxia-induced angiogenesis, suggesting that there is a long-term adaptive response to metabolic challenges (Harb et al., 2013). In line with this, several studies in animal models showed that CBF depletion induces and/or intensifies neuronal dysfunction and/or AD-like neuropathological changes (Sagare et al., 2012). In fact, it was reported that mild hypoperfusion increased the neuronal Aβ concentration and the levels of phosphorylated tau in an AD mouse model (Koike MA, 2010). It was also observed that brain ischemia in rodent lead to increased levels of hyperphosphorylated tau in neurons and filaments similar to those observed in human AD tauopathy (Gordon-Krajcer et al., 2007). Moreover, arterial carotid occlusion in rats lead to neuronal dysfunction, including synaptic changes (Wang et al., 2010a). These changes are major contributing factors to age-related neurovascular vulnerabilities and are currently under intense investigation as potential therapeutic targets. A recent study already proved that factors found in young blood, namely GCF11, induce vascular remodeling and associated neurogenesis, as well as improved olfactory discrimination in aged mice (Katsimpardi et al., 2014). Despite all of this evidence, little is known about the vascular factors that separate age-dependent memory impairment from AD. In addition, the relationship between age-related memory impairment and CBF reduction is not clearly understood.

2.2 Age-related neuronal and glial alterations

Altered synaptic morphology, progressive loss of synapses and glial (astrocyte and microglial) cell activation are considered characteristic hallmarks of aging (Ojo et al., 2012). Here, some key aspects that are involved are schematically depicted in figure 5.

2.2.1 Neuronal impairment in aging

A common misunderstanding about the brain aging process is that the functional failure observed in some individuals is just a reflection of intense neuronal death. In healthy aging individuals, brain activation is more anterior, less lateralized and more coordinated than in those at risk of, or suffering from, cognitive impairment (Topiwala and Ebmeier, 2012). One of the most exciting theories to attempt to explain this is the scaffolding theory that states that the older brain is a plastic homeostatic organ, able to compensate for its deteriorating structure (Topiwala and Ebmeier, 2012). This explains why there are some regions in the older brains that, surprisingly, are more active than in the younger counterparts.

Interestingly, a recent study showed that most brain structures do not follow a simple path throughout adult life and that accelerated decline in old age is not the norm of healthy brain aging (Fjell et al., 2013).

Historically, it was believed that aging was marked by massive nerve cell loss, decreased neuronal activation and an absence of the plasticity that mediates skill acquisition. However, more recent studies have changed the overall consensus. Several studies of aging in rhesus monkey, rat and humans found a minimal, if any, loss of excitatory or inhibitory neurons in cortical regions and in the hippocampus (Hof and Morrison, 2004; Morrison and Baxter, 2012). These results are intriguing in the way that age-dependent memory impairment could be associated with other events and not only neuronal loss in hippocampus. On the other hand, other studies revealed that in other brain regions, like the cerebellum and the substantia nigra, aging is accompanied by some neuronal loss, whereas AD is characterized by a more marked neuronal depletion in region CA1 of the hippocampus (Andersen et al., 2003; Woodruff-Pak et al., 2010). The mechanisms responsible for age-related cognitive impairment remain unclear, and it is still difficult to reconcile cognitive decline with the neuropathological process. Therefore, it is important to understand whether vascular factors and abnormal vascular functioning trigger and/or accelerate the neuropathological process. Interleukin (IL)-6 overproduction by astrocytes and low-grade microglial neuroinflammation may contribute to the modification of neuronal functioning during aging (Sauvant et al., 2014). The activation of caspase-6 in mouse CA1 neurons is a sufficient event to induce neuronal degeneration ad age-dependent memory impairment, suggesting that lower cognitive performance of aged humans could be related to it and targeting caspase-6 could be a potential treatment strategy for AD (LeBlanc et al., 2014). In addition, knockdown of Nfr2 resulted in reduced proteasome activity, resistance to stress and longevity, whereas prolonged overexpression of it resulted in reduced longevity, indicating that proteostasis could be fundamental to accelerated brain aging (Tsakiri et al., 2013). To identify the proteomic changes along aging, a recent study demonstrated that extracellular matrix proteins were the only group of proteins that presented a consistent and progressive upregulation over time (Vegh et al., 2014). Thus, it is possible that increased levels of hippocampal extracellular matrix proteins may limit synaptic plasticity, and as such, might be a potential target to modulate age-dependent cognitive impairment (Vegh et al., 2014). Additionally, another study reported age-related decline in mitochondrial activity, reduced antioxidant contents, increased oxidative stress markers and increased sensitivity to Aβ toxicity in resting and depolarized synaptic terminals (Quiroz-Baez et al., 2013). Structural changes in neurons and dendritic spines, as well as alterations in neurotransmitter receptors and in electrophysiological properties, were found in normal aging (Duan et al., 2003; Hof et al., 2002). It is interesting also to relate the changes observed along normal aging, MCI and AD. Several studies used stereological techniques and found that in MCI there is a significant loss

of neurons in the frontal cortex, the entorhinal cortex and the CA1 field of the hippocampus (Price et al., 2001; Scheff et al., 2007)

AD presents neuronal dysfunction that seems to be related to Aβ and tau. Deposits of Aβ protein are apparent with aging in several species, such as non-human primates, dogs and bears, and others (Finch and Austad, 2012; Head, 2011; Mutsuga et al., 2012). Studies of transgenic mouse models of AD have shown that there is an abrupt spine loss and neurite dystrophy in the neighborhood of amyloid plaques (Spires-Jones et al., 2007; Spires et al., 2005; Tsai et al., 2004). Also soluble Aβ was shown to induce dendritic spine loss (Selkoe, 2008). However, little is known about the mechanism through which Aβ promotes synaptic loss and neuronal death, and if these changes in neuronal dendritic branching and neurites occur before, simultaneously or after the Aβ accumulation. The same questions remain regarding the temporal relationship between the neurodegeneration process, changes in BBB and alterations in vascular density within this multifactorial process.

2.2.2 Glial dysfunction in aging and contribution to neurodegeneration

Microglia are brain-resident immunocompetent cells. In response to homeostatic disturbance of the CNS they change their phenotype leading to signaling transduction activation and secretion of cytokines and vasoactive molecules that regulate BBB permeability and CBF (Nimmerjahn et al., 2005). Microglia cells from human aged brains present clear alterations, characterized by dystrophic processes and abnormal clustering (Streit et al., 2008). Increased microglial activation has been reported in aged humans (Dickson et al., 1992). In the course of healthy aging of nonhuman primate brain, the expression of the major histocompatibility complex class II is increased (Sheffield and Berman, 1998) and microglial phagocytic activity increased age-dependently (Peters et al., 1991). In addition, a previous study reported that pro-inflammatory cytokines are increased and anti-inflammatory cytokines are decreased in aged mouse brain (Ye and Johnson, 2001), which further indicates an abnormal state of microglia in aging. Little is known about what triggers the microglia activation in a healthy aging brain, and more research in this area would be useful to understand some related age-dependent pathological processes. Feor example, it is possible that the loss of some endogenous factors responsible for the anti-inflammatory response in aging brain may be involved. Recent data showed that neuronal genotoxic stress is sufficient to switch microglia from a resting to a primed state, characterized by an exaggerated response to peripheral lipopolysaccharide exposure, both in terms of cytokine expression and phagocytosis (Raj et al., 2014). In normal aging the expression of RAGE is increased, but it is interesting that RAGE-expressing microglia are even further augmented in AD brain. This receptor is known to mediate the pro-inflammatory effect of Aβ, accelerating or amplifying the inflammatory response,

leading to recruitment or activation of microglia and astrocytes (Fang et al., 2010). Synaptic depression induced by oxygen glucose deprivation was enhanced by the presence of Aβ, however, when RAGE was inhibited, that depression was ameliorated (Origlia et al., 2014). As such, RAGE inhibition may be protective against the toxic effects of oxygen glucose deprivation, in an amyloid-enriched environment. Taken together, this means that the RAGE-dependent neuroinflammatory pathway plays an important role in synaptic failure in diseases like AD, which makes this pathways a potential therapeutic target. Moreover, microglial behavior during aging may be explained in part by the fact that during aging, the classical microglia activation pathway involving tumor necrosis factor–α, IL–12 and IL-1β pathways is enhanced, the alternative activation pathway, including IL-4/ IL-13 signaling, is reduced (Lee et al., 2013). Moreover, it has been shown that microglia cells in the neocortex exhibit age-related soma volume increase, increased soma movement, shortening of processes, decreased process speed, as well as loss of homogenous tissue distribution (Hefendehl et al., 2014). Altogether, these data show that microglia dysfunction along aging strongly contributes to age-related vulnerabilities and may increase the risk of progression to AD-like pathologies. Age-related changes in AD microglia are more prevalent than in age-matched brains from non-demented individuals (Flanary et al., 2007), particularly in early AD pathogenesis, a phase in which microglial activation may have beneficial effects by scavenging and clearing toxic Aβ from the brain (Bell and Zlokovic, 2009). However, in latter stages of the disease, microglia do not appear to respond to normal regulatory feedback (Sawada et al., 2007) and become unable to eliminate Aβ and to cope with toxic compounds (Bell and Zlokovic, 2009; Flanary, 2005) (Sawada et al., 2007). As a result of microglia activation in AD, there is an increased exposure of brain cells to cytokines, chemokines, MMPs and, the promotion of astrocytic chemotaxis around senile plaques (Glass et al., 2010), where activated microglia have been found as well (Suzumura, 2009). Activated microglia also contribute to neuronal hyperexcitability in AD, as they release excessive quantities of glutamate (Suzumura, 2009), which may further contribute to neurodegeneration. Despite the recognition of the important role of microglia in AD progression, much remains unknown regarding microglia phenotypic changes, as well as how age-associated microglia activation contributes to AD pathogenesis.

Astrocytes also present age-related changes and contribute to neurodegeneration in AD. Although several astrocytic functions have been shown to be affected by aging, including synaptic plasticity and metabolic balance, astrocytic changes have received much less attention than neuronal alterations. The messenger ribonucleic acid expression of glial fibrillar acidic protein and S100B (markers for activated astrocytes) is higher in aged brain (Godbout and Johnson, 2009), revealing an increased activated state. While in the aging brain astrocytes are increased in number by about 20% (Minagar et al., 2002), in AD they are also increased in size and motility and are located in close

proximity to senile plaques (Salminen et al., 2011). This localization around senile fibrillar amyloid plaques suggests that Aβ deposition triggers astrocyte activation in AD brain (Carrero et al., 2012), it may also be possible that Aβ deposits (or reaction to them) triggers a signal to recruit astrocytes to plaques and then they become activated. Interestingly, cytokines and Aβ increase the levels of astrocytic BACE1, APP, and Aβ, and stimulate amyloidogenic APP processing in astrocytes; given that astrocytes greatly outnumber neurons, activated astrocytes may thus represent a significant source of Aβ by this feed-forward mechanism (Zhao et al., 2011). Astrocyte behavior during aging is modified in APOE4 old mice, in which there is less microglial clearance of Aβ, leading to more intraneuronal accumulation; moreover, it was observed that decreased clearance leads to more extracellular Aβ, and more downstream consequences relating to astrocyte activation and phospho-tau accumulation (Zhao et al., 2014). Also, astrocytes may play a role in the regulation of calcium signaling during aging. It was found that astrocytic expression of calpain-10 is upregulated, and CamKIIα is downregulated with increasing Braak AD staging, while calpain-10 immunoreactivity is correlated with both local and global measures of Alzheimer-type pathology (Garwood et al., 2013). Astrocytes present an age-related increase of estrogen receptor-α, which contributes to the estradiol desensitization of the neuronal responses and impairs neurotrophic support (Arimoto et al., 2013). Moreover, astrocyte and microglia activation is correlated with decreased myelin proteins in aging brain, which reveals a new link between inflammation and myelin breakdown (Xie et al., 2013). Recent findings in rat aged brains showed that the astrocytic expression of the efflux receptor LRP-1, is decreased and the influx receptor RAGE is increased (Silverberg et al., 2010a; Silverberg et al., 2010b), thus increasing the intracellular accumulation of their substrates, including Aβ. Another aspect that is affected by age is neurogenesis. In fact, the ability of astrocytes to protect neurons and to promote neurogenesis is decreased in an aged brain (Miranda et al., 2012). Recently, a signaling pathway was discovered in which astrocytes regulate hippocampal neurogenesis, but whether this pathway declines with aging remains to be determined (Ashton et al., 2012). Thus, despite all the studies to understand the astrocyte function in the brain, the functional and cellular response of astrocytes to AD pathogenesis remains unclear. Astrocytes are the main producers of APOE in CNS, an apolipoprotein playing a key role in Aβ transport in the CNS, as already described earlier in this review. In the aging brain, Aβ accumulation seems to be related to the astrocytic increased production of APOE (Bien-Ly et al., 2012). Taken together, all of these findings suggest that astrocytes play an important role in AD that still needs to be clarified.

Conclusion and future perspectives

Despite tremendous progress in our current knowledge regarding the mechanisms underlying neurodegeneration, many important questions remain unanswered. Therefore, further efforts should be undertaken in order to clarify how aging predisposes to neurodegenerative diseases in general, and to AD in particular, to decipher the contribution of the different players in the orchestra of neurodegeneration, and to discern the contribution of vascular dysfunction in the pathogenesis of neurodegenerative diseases. Hopefully, clarification of these issues will reveal reliable biomarkers to predict individuals at risk, and identify novel targets to avoid disease occurrence or, at least, delay disease progression. Moreover, a better comprehension of the cellular alterations and interplay within the neurovascular unit during aging and neurodegenerative disease pathogenesis, as well as of the signaling pathways at a cellular level, is pivotal for encouraging novel therapies to prevent or counteract neurodegenerative diseases, including AD.

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3. Aims

The pathogenesis of AD is still unclear, with the amyloid theory stating that neuronal loss and cognitive decline are a result of Aβ cascade, whereas the vascular theory states that vascular dysfunction precedes, accelerates or contributes to the progression of amyloidopathy and neurodegeneration. On the other hand, the factors that predispose the aged brain to AD have still not been clarified and the relevance of BBB properties impairment in aging is still obscure. Therefore, with this project we aimed to (1) investigate which vascular and glial events are characteristic of AD or/and aging, (2) establish the temporal evolution of these changes in AD-like APP/PS1 and wild-type (WT) mice, and (3) relate these changes with Aβ accumulation. To this end, we will use the AD-like mice model APP/PS1ΔE9, that allows us evaluate the disease progression, and the C57BL/6 wild-type (WT) mice, in order to analyze the alterations during aging. We will establish the temporal sequence of vascular and glial changes and relate them with the appearance of senile plaques. Thus, we will contribute to clarify the contributing events to the pathogenesis of AD and to better understand why is aging a predisposing factor for AD.

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Chapter II

Glio-vascular changes during aging in wild-type and in Alzheimer's disease-like APP/PS1 mice

The work presented in this chapter originated the following manuscript:

Glio-vascular changes during aging in wild-type and in Alzheimer's disease-like APP/PS1 mice
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European Journal of Neurosciences (Submitted)

Abstract

Vascular and glial involvement in the development of neurodegenerative disorders, such as Alzheimer's disease (AD), and age-related brain vulnerabilities has been suggested. Therefore, we sought to: i) investigate which vascular and glial events are evident in aging and/or AD, ii) to establish the temporal evolution of vascular and glial changes in AD-like and wild-type (WT) mice and iii) to relate them to amyloid-β (Aβ) accumulation. We examined immunohistochemically hippocampi and cortex from APP/PS1dE9 and WT C57BL/6 mice along aging and disease progression (young-adulthood, middle- and old-age). Aging resulted in the increase in receptor for advanced glycation endproducts and desmin expression, as well as the entrance of thrombin and albumin in hippocampus parenchyma. In contrast, the loss of platelet-derived growth factor receptor-β (PDGFR-β) positive cells, in both regions, was only related to AD pathogenesis. Hypovascularization was affected by both aging and AD in the hippocampus, but resulted from the interaction between both factors in the cortex. Astrogliosis was a result of AD in hippocampus and by both factors in cortex, while microgliosis was associated with fibrillar amyloid plaques in AD-like mice and with the interaction between both factors in each of the studied regions. In sum, these data show that senile plaques precede vascular and glial alterations just in hippocampus, whereas in cortex, vascular and glial alterations, namely loss of PDGFR-β-positive cells and astrogliosis, accompanied the first senile plaques. Hence, this study points to vascular and glial events that co-exist with AD pathogenesis and age-related brain vulnerabilities.

Keywords: Blood-brain barrier disruption, glial activation, microvasculature, pericytes, receptor for advanced glycation endproducts

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by intracellular neurofibrillary tangles of hyperphosphorylated tau (tauopathy), amyloid-β protein (Aβ) extracellular aggregates (amyloidopathy), oxidative stress, inflammation and premature neuronal apoptosis (Serrano-Pozo et al., 2011). In the last few years, the relationship between the accumulation of Aβ, neuronal atrophy, glial activation and vascular dysfunction has been the target of multiple studies, as evidence grows to suggest that all of these events may possibly contribute to development of AD (Armstrong, 2013). Multiple studies have shown that cerebrovascular dysfunction, namely blood-brain barrier (BBB) disruption, and cerebral blood flow and capillary density alterations, may lead to faulty Aβ clearance from the brain (Deane et al., 2004a; Deane et al., 2004b), augmented influx of peripheral Aβ through the BBB (Eisele et al., 2010) and overexpression of amyloid precursor protein (APP) (Kumar-Singh et al., 2005; Weller et al., 2008). Thus, more studies are required to understand the temporal relationship between vascular damage and glial activation, and whether neuronal dysfunction begins before or after the accumulation of Aβ and vascular damage. Furthermore, aging is known to contribute to the compromise of BBB properties and to alterations of the microvasculature, which in turn contribute to age-related cognitive decline possibly due to their influence on neuronal and glial activity (Marques et al., 2013). Recently, a study of elderly individuals with normal cognition to mild dementia manifesting vascular disease showed that vascular brain injury was more influential than Aβ to mild cognitive dysfunction (Marchant et al., 2013), which is particularly interesting because AD is not a vascular dementia. Hence, considering that aging is the greatest risk factor for AD (Akinyemi et al., 2013) it is important to understand which are the age-associated brain alterations, in order to modulate or prevent them, and to the severity of age-related brain vulnerabilities.

In this study we sought to establish the glial and vascular events that are prevalent in aging and/or AD. Moreover, we wanted to understand how glial and vascular profiles evolve during aging and AD progression. The results presented here reveal vascular and glial alterations occurring along aging, thus contributing to a better understanding of age-related brain vulnerabilities. Moreover, by establishing the temporal sequence of events occurring in AD and identifying the early ones, this study points to potential therapeutic targets to counteract AD development and progression.

2. Materials and methods

2.1 Animals

In this study, brain sections of APPswe/PS1ΔE9 transgenic mice and C57BL/6 wild-type (WT) mice were immunohistochemically examined at each of three different ages: 6 month (young adults, n=

5), 12-14 month (middle age, n=5), and 23-28 month (old, n=4). All animal use was approved by the Harvard Standing Committee for Animal Use and was in compliance with all state and federal regulations. APPswe/PS1ΔE9 (hereafter designated as APP/PS1) mice are carriers of the Swedish APP (K594N/M595L) and PS1ΔE9 (deletion of exon 9) human transgenes under the promoter of a mouse prion protein (Jankowsky et al., 2004). APP/PS1 mice mimic to some degree early-onset human AD (Xiong et al., 2011). This mouse model starts developing occasional Aβ deposits by 5-6 months of age (Jankowsky et al., 2004), presenting abundant plaques in cortex and hippocampus at eight months, which increase along disease progression, leveling off after 18 months of age (Garcia-Alloza et al., 2006; Wirz et al., 2013), as shown in Supplement Figure 1. Whereas there are several studies showing that cognitive deficits manifest at 6 months and are exacerbated with increasing amyloid burden (Gimbel et al., 2010; Park et al., 2006; Xiong et al., 2011), there are other studies demonstrating that these animals present impaired spatial learning starting at 12 months in the Morris water maze and, that 13 months-old APP/PS1 mice commit more errors in the same test than WT mice, unlike at 7 months wherein there is no difference (Lalonde et al., 2005). Deficits in transient electrophysiological recordings of long-term potentiation are observed as early as 3 months and this impairment is not related to age from 3 to 12 months (Volianskis et al., 2010).

2.2 Tissue collection

All mice were killed by CO_2 inhalation and transcardially perfused with 20 ml phosphate buffer saline. The brain of each mouse was removed and divided sagittally. As previously described, (Maier et al., 2005) the brain was fixed for 2 h in 10% buffered formalin before being processed for paraffin embedding.

2.3 Immunohistochemistry

Immunohistochemistry was performed on 10 μm paraffin sections of mouse brain as previously described (Lemere et al., 2000), and summarized in Table 1. A 0.3% hydrogen peroxide in methanol solution was used to inhibit endogenous peroxidase, for 10 minutes, and antigen recovery was achieved by heat-mediated treatment with citrate buffer. Sections were incubated overnight at 4ºC with the primary antibodies, and then incubated for 30 minutes with secondary antibodies. The Vectastain Elite ABC kits was used (Vector Laboratories, Burlingame, CA), followed by development with 3,3'-diaminobenzidine tetrahydrochloride. Hematoxylin counterstaining was performed for some of the immunostainings. Negative controls with omission of primary antibodies were performed to exclude nonspecific binding or cross reactivity.

Table 1. Summary of the antibodies and experimental conditions used for immunohistochemical analysis.

	Markers	T/NT	Blocking	Primary antibody	Dilution	Secondary antibody	Dilution
Vascular	CD31	NT	5% GS, Triton 0.05%	Abcam, #ab28364 Rabbit Pc	1:50 in 5% GS	Vector, #BA-1000 Biotinylated Goat anti-Rabbit	1:2000 in 10% GS
	Albumin	NT	5% HS	Bethyl, #A90-134A Goat Pc	1:100 in 5% HS	Vector, #BA-9500 Biotinylated Horse anti-Goat	1:2000 in 10% HS
	Thrombin	T	2% HS	Santa Cruz, #sc-23335 Goat Pc	1:100 in 2% HS	Vector, #BA-9500 Biotinylated Horse anti-Goat	1:2000 in 10% HS
	Desmin	T	2% GS	DAKO, #M0760 Mouse Mc	1:100 in 2% GS	Southern Biotech, #1070-08 Biotinylated Goat anti-Mouse IgG1	1:2000 in 10% GS
	PDGFR-β	T	2% GS	R&D, #AF1042 Goat Pc	1:20 in 2% HS	Vector, #BA-9500 Biotinylated Horse anti-Goat	1:2000 in 10% HS
	RAGE	T	2% GS	NeoBioLab, #A1786 Rabbit Pc	1:350 in 2% GS	Vector, #BA-1000 Biotinylated Goat anti-Rabbit	1:2000 in 10% GS
Glial	GFAP	NT	10% GS	Dako, #Z033429-2 Rabbit Pc	1:500 in 10% GS	Vector, #BA-1000 Biotinylated Goat anti-Rabbit	1:2000 in 10% GS
	Iba-1	T	10% GS	Wako, #019-19741 Rabbit Pc	1:500 in 10% GS	Vector, #BA-1000 Biotinylated Goat anti-Rabbit	1:2000 in 10% GS
	Amyloid-β	T	10% GS	Dr .Dennis Selkoe Lab Rabbit Pc	1:1000 10% GS	Vector, #BA-1000 Biotinylated Goat anti-Rabbit	1:2000 in 10% GS

CD31, cluster of differentiation 31; PDGFR-β, platelet derived growth factor receptor β; RAGE, receptor for advanced glycation endproducts; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor molecule 1. # = catalog reference; Pc, polyclonal antibody; Mc, monoclonal antibody; T, pretreatment with antigen retrieval by microwave treatment using citrate buffer; NT, no pretreatment with antigen retrieval by microwave treatment using citrate buffer; GS, goat serum; HS, horse serum.

2.4 Data analysis

Photographs were acquired on bright field microscope (Zeiss, model AxioSkop HBO50) with an integrated digital camera (Leica, model DFC490). Twelve fields of the hippocampus and twelve fields of cortex of each animal were analyzed using the ImageJ 1.29x software (National Institutes of Health, USA), for glial and vascular indicators, as detailed below. Vascular density, receptor for advanced glycation endproducts (RAGE) immunoreactivity by endothelial cells, desmin and platelet derived growth factor receptor-β (PDGFR-β) expression by pericytes and blood-borne components in brain parenchyma were determined to assess vascular alterations. For all of these vascular parameters, only capillaries (i.e., blood vessels with a diameter lower than 6 μm) were considered. For the vascular density evaluation, the area of cluster of differentiation (CD) 31-positive capillaries per field was measured (Brito et al., 2013), considering both longitudinal and transversal blood vessels, and data was expressed as total

area of blood vessels per μm^2 of tissue. For evaluation of RAGE immunoreactivity in endothelial cells, each longitudinal capillary was delimited, the vascular immunoreactivity was measured, and results were expressed by the mean intensity of RAGE per μm^2 of blood vessel. To evaluate the number of pericytes expressing PDGFR-β, a widely used marker for these cells (Bell et al., 2010; Sá-Pereira et al., 2012), the number of total PDGFR-β-positive cells per field was counted. Moreover, the number of perivascular pericytes was evaluated by measuring the number of PDGFR-β-positive cells per longitudinal blood vessel. The blood vessels were then classified into three categories: blood vessels without or blood vessels with one or with two or more PDGFR-β-positive cells. Pericytes were further evaluated based on the expression of desmin, another commonly used pericyte marker (Bell et al., 2010; Sá-Pereira et al., 2012), and the total desmin-positive area (μm^2) per blood vessel was determined in longitudinal vessels. To evaluate the entrance of thrombin and albumin into the brain parenchyma, widely used markers of BBB hyperpermeability (Bell et al., 2010; Brito et al., 2013), the number of thrombin-positive and albumin-positive deposits per section was measured. Glial parameters were analyzed based on the widely used astrocytic and microglial markers, glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1), respectively. To evaluate the density of both glial cells, the number of GFAP- and Iba-1-positive cells per field was counted. Moreover, the number of perivascular glial cells was evaluated by counting the number of GFAP- and Iba-1-positive cells per longitudinal capillaries. The capillaries were then classified into three categories: blood vessels without or with one, two or more GFAP- or Iba-1-positive cells. Aβ burden in brain parenchyma was previously determined by Lemere lab (Frost et al., 2013) for each mouse in the present study through semi-quantitative scoring of Aβ plaques per section.

2.5 Statistical analysis

Results were analyzed using GraphPad Prism® 5.0 (GraphPad Software, San Diego, CA, USA) and are expressed as mean±SEM. One-way ANOVA and the Bonferroni post hoc test were used to evaluate how the parameters evolved along time for each genotype separately. Two-way ANOVA was used to evaluate the difference between WT and APP/PS1 mice at each time point, as well as to determine how the parameters were affected by aging and AD. Two-way ANOVA F values are presented. P values less than 0.05 were considered statistically significant.

3. Results

3.1 Endothelial changes during aging in WT and in AD-like APP/PS1 mice

Based on previous studies suggesting that aging of healthy individuals and AD are both accompanied by alterations in endothelial cells (Farkas and Luiten, 2001), we investigated the temporal evolution of blood vessel density (Fig 1) and microvascular RAGE immunoreactivity (Fig 2) in hippocampus and cortex of WT and APP/PS1 mice. Analysis of the microvessel density of WT animals showed a 35% ($P<0.05$) and 46% ($P<0.05$) decrease from middle-age to old age in hippocampus (Fig 1B) and cortex (Fig 1C), respectively. Regarding APP/PS1 animals, no significant loss of microvessel density in hippocampus was observed along time (Fig 1B), while in cortex a significant reduction of 40% from young adulthood to middle-age was observed ($P<0.01$, Fig 1C). Therefore, these results show that the loss of microvascularization occurs earlier in cortex in AD (from young adulthood to middle-age) than in WT (from middle to old age) mice. This fact supports that the biggest difference between both genotype groups was observed by middle-age, where APP/PS1 mice had significantly less ($P<0.01$ in hippocampus and $P<0.05$ in cortex) microvessel density than WT mice. Remarkably, by old age both APP/PS1 and WT mice presented similar microvessel density in both regions (Fig 1B, C). Thus, both aging and AD contributed to hypovascularization in hippocampus (two-way ANOVA F 6.012, $P<0.05$ and F 13.86, $P<0.01$, respectively), whereas in cortex it was a result of the interaction between aging and AD (two-way ANOVA interaction AD × aging F 3.733, $P<0.05$).

Analysis of RAGE immunoreactivity by endothelial cells of WT animals showed an increase over time, and that this increase was already apparent at middle age in the hippocampus (24% vs. young adult, $P<0.01$, Fig 2B), and only at old age in the cortex (21% vs young adult, $P<0.05$, Fig 2C). The APP/PS1 animals also showed a 13% increase in RAGE immunoreactivity in hippocampus ($P<0.05$ young adulthood vs. middle-age, Fig 2B) and, even though not significant, a 14% increase was observed in cortex (young adult vs. middle-age, Fig 2C). Comparison of endothelial RAGE immunoreactivity between WT and APP/PS1 animals showed no difference in both regions. Accordingly, aging was the major contributor for the alterations in RAGE immunoreactivity by endothelial cells in hippocampus (two-way ANOVA F 23.13, $P<0.001$) and cortex (two-way ANOVA F 9.874, $P<0.001$). In summary, endothelial-related changes indicate that APP/PS1 mice have a premature loss of capillary density compared to WT mice, but similar immunoreactivity of RAGE by endothelial cells with aging.

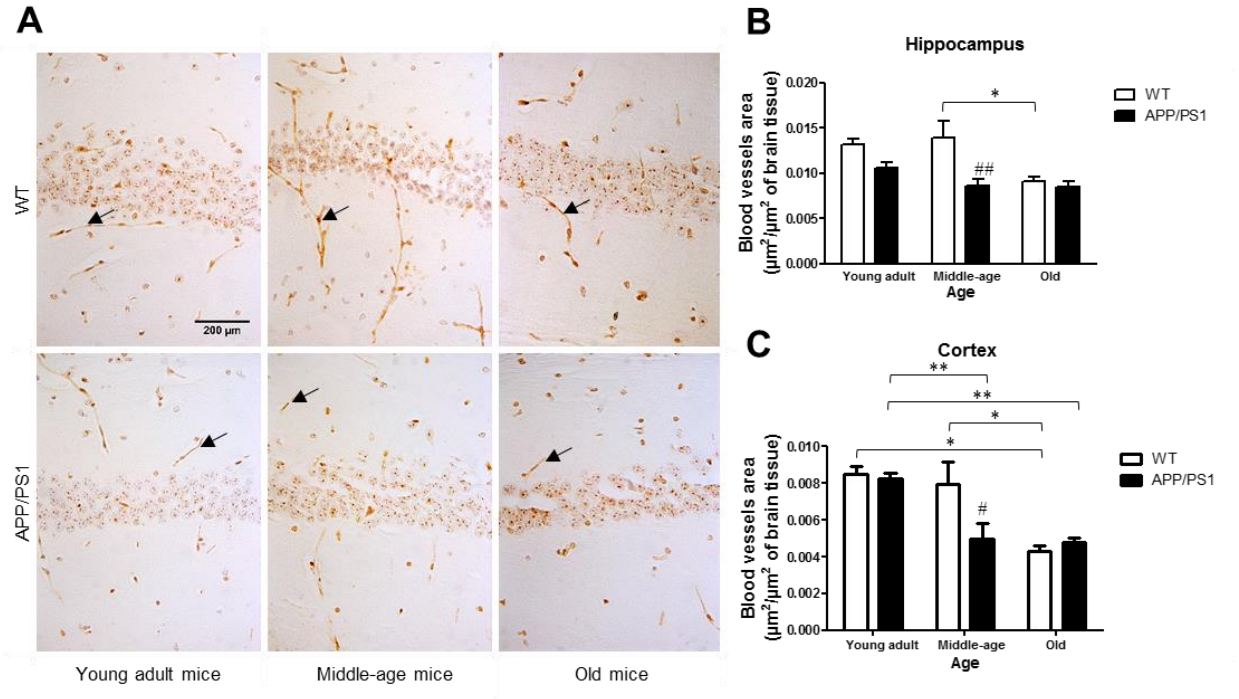

Fig. 1. Microvascular density changes associated with Alzheimer's disease (AD) and aging in hippocampus and cortex. Brain sections of APP/PS1 transgenic mice, a mouse model of AD, and C57BL/6 wild-type (WT) mice at 6, 12-14 and 23-28 months (young adulthood, middle-age and old mice, respectively) were processed for immunohistochemical analysis of the endothelial marker, cluster of differentiation 31 (CD31). Representative immunohistological patterns of CD31 in hippocampus (A), where arrows point to CD31 positive microvessels. Semi-quantitative analysis of the area of blood vessels per brain area in hippocampus (B) and cortex (C). $^{*}P<0.05$, $^{**}P<0.01$ between indicated groups; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. age-matched WT.

3.2 Pericyte alterations during aging in WT and in AD-like APP/PS1 mice

We wanted to know if aging and/or AD affect blood vessels integrity by altering the expression of pericytes markers (Fig 3, 4). For that purpose, we analyzed PDGFR-β-positive cells (Fig 3A), and the expression of desmin (Fig 4A) along aging and/or AD. Analysis of PDGFR-β-positive cells in hippocampus (Fig 3B) and cortex (Fig 3C) showed that in cortex there was nearly triple the number of PDGFR-β-positive cells per field than in the hippocampus. WT mice did not show a significant alteration of the number of positive cells in either brain region with aging, and the same was observed in APP/PS1 mice (Fig 3B, C). When WT were compared with APP/PS1 mice at each time point, no significant difference was observed in the hippocampus (Fig 3B), whereas in the cortex APP/PS1 mice had fewer immune-labeled cells than WT (Fig 3C). In fact, by young adulthood APP/PS1 had already 32% ($P<0.01$) fewer PDGFR-β-positive cells in cortex than WT mice (Fig 3C), a difference that was preserved during middle-age ($P<0.01$). Accordingly, the major factor contributing to the loss of PDGFR-β in hippocampus and cortex was not aging, but AD (two-way ANOVA $F<14.95$, $P<0.001$ and $F<27.31$, $P<0.001$, respectively).

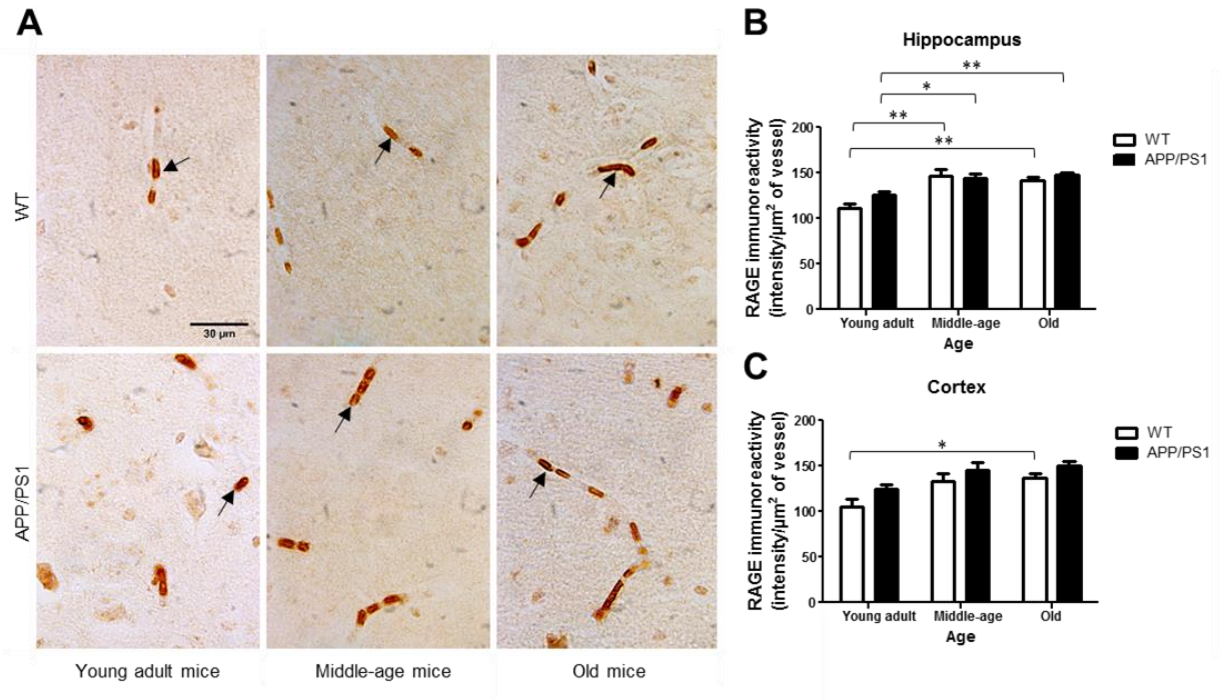

Fig. 2. Endothelial receptor for advanced glycation end products (RAGE) changes associated with Alzheimer's disease (AD) and aging in hippocampus and cortex. Brain sections of APP/PS1 transgenic mice, a mouse model of AD, and C57BL/6 wild-type (WT) mice at 6, 12-14 and 23-28 months (young adulthood, middle-age and old, respectively) were processed for immunohistochemical analysis of endothelial immunoreactivity of RAGE. Representative immunohistological pattern of RAGE in hippocampus (A), where arrows point to RAGE positive microvessels. Semi-quantitative analysis of RAGE immunoreactivity in endothelial cells in hippocampus (B) and cortex (C). $^{*}P<0.05$, $^{**}P<0.01$ between indicated groups.

In addition to the total number of PDGFR-β-positive cells, we investigated the perivascular location of these cells. Even though a significant age-related loss of total pericytes was not observed in either hippocampus (Fig 3B) or cortex (Fig 3C), it is possible that the PDGFR-β-positive cells may have dissociated from vessels along aging in both regions, since the number of blood vessels not associated with pericytes increased from ~20% to ~40% in hippocampus ($P<0.05$, from young adult to old mice) and from ~30% to ~45% in cortex ($P<0.01$, from middle-age to old mice) in WT mice (Fig 4D,E, respectively). In accordance, the number of blood vessels with two or more PDGFR-β-positive cells declined from ~30% to ~15% in hippocampus and from ~20% to ~10% in cortex ($P<0.01$, from young adult to old mice. Analysis of APP/PS1 mice showed that while the cortex does not present alterations in the perivascular PDGFR-β-positive cells along disease progression (Fig 3E), the hippocampus (Fig 3D) manifests a significant decrease of pericytes associated with endothelial cells during disease development, since the number of blood vessels with no pericytes increased from ~30% to ~50% ($P<0.01$, from young adult to middle-age mice). Comparison of WT with APP/PS1 animals showed a

significant difference in the number of PDGFR-β-positive cells per blood vessel in cortex in young adulthood, whereas APP/PS1 mice had ~25% ($P<0.001$) more blood without pericytes and a decline of 15% ($P<0.05$) of blood vessels with two or more pericytes (Fig 3E). In the hippocampus, APP/PS1 had ~13% less blood vessels with two or more cells, albeit, this difference did not reach statistical significance. In sum, in cortex of APP/PS1 there was both a significant loss of total pericytes and perivascular PDGFR-β-positive cells when compared to age-matched WT, whereas in the hippocampus there was not this a significant difference in the total number pericytes, although we observed a more robust decrease of perivascular cells in APP/PS1 mice compared to WT. Furthermore, desmin-positive cells were analyzed (Fig 4A) and the area per blood vessel was measured (Fig 4B,C). It is noteworthy to mention that in hippocampus (Fig 4B), desmin-positive area per blood vessel was almost the double that observed in cortex (Fig 4C). Analysis of WT animals showed that there was a significant increase of 39% ($P<0.05$, from young adulthood to middle-age, sustained thereafter) in hippocampus (Fig 4B), while in cortex there was not a significant age-dependent increase (Fig 4C). APP/PS1 mice revealed a significant increase in desmin-positive staining per blood vessel with aging, including an increase of 30% ($P<0.05$, from young adulthood to middle-age) in hippocampus (Fig 4B) and of 37% ($P<0.05$, from young to old mice) in cortex (Fig 4C). Since these alterations were a result of aging in both regions (two-way ANOVA F 12.94, $P<0.001$ in hippocampus; F 6.971, $P<0.01$ in cortex), and not AD, is understandable why we did not observed any difference between WT and APP/PS1 animals. Thus, these results show that while the expression of PDGFR-β is mainly affected by AD, the expression of desmin is only affected by aging.

3.3 Entrance of blood-borne components into hippocampal parenchyma during aging in WT and in AD-like APP/PS1 mice

Based on previous studies showing that pericytes play a key role in the maintenance of the BBB integrity (Bell et al., 2010; Sá-Pereira et al., 2012), we investigated if the loss of perivascular pericytes is accompanied by the entrance into the brain parenchyma of thrombin and albumin (Fig 5), two blood-borne components widely used as indicators of BBB disruption (Bell et al., 2010; Brito et al., 2013). Interestingly, positive deposits of these proteins were only detected in the hippocampus (Fig 5A, C), which is the region presenting the lowest number of pericytes as shown by the lower number of PDGFR-β-positive cells per field (Fig 3 B,C). An ~18-fold increase was detected in WT mice, while a ~40-fold increase ($P<0.01$) was observed in APP/PS1 mice in the number of albumin-positive deposits from young adulthood to old mice (Fig 5B). In line with these results showing that albumin-positive deposits were mainly detected in the old mice, aging was the only factor affecting the entrance of albumin (two-way ANOVA F 13.21, $P<0.001$). The number of thrombin-positive deposits increased ~10-fold for WT and APP/PS1 mice from young adulthood to middle-age, further increasing in old animals (Fig 5D).

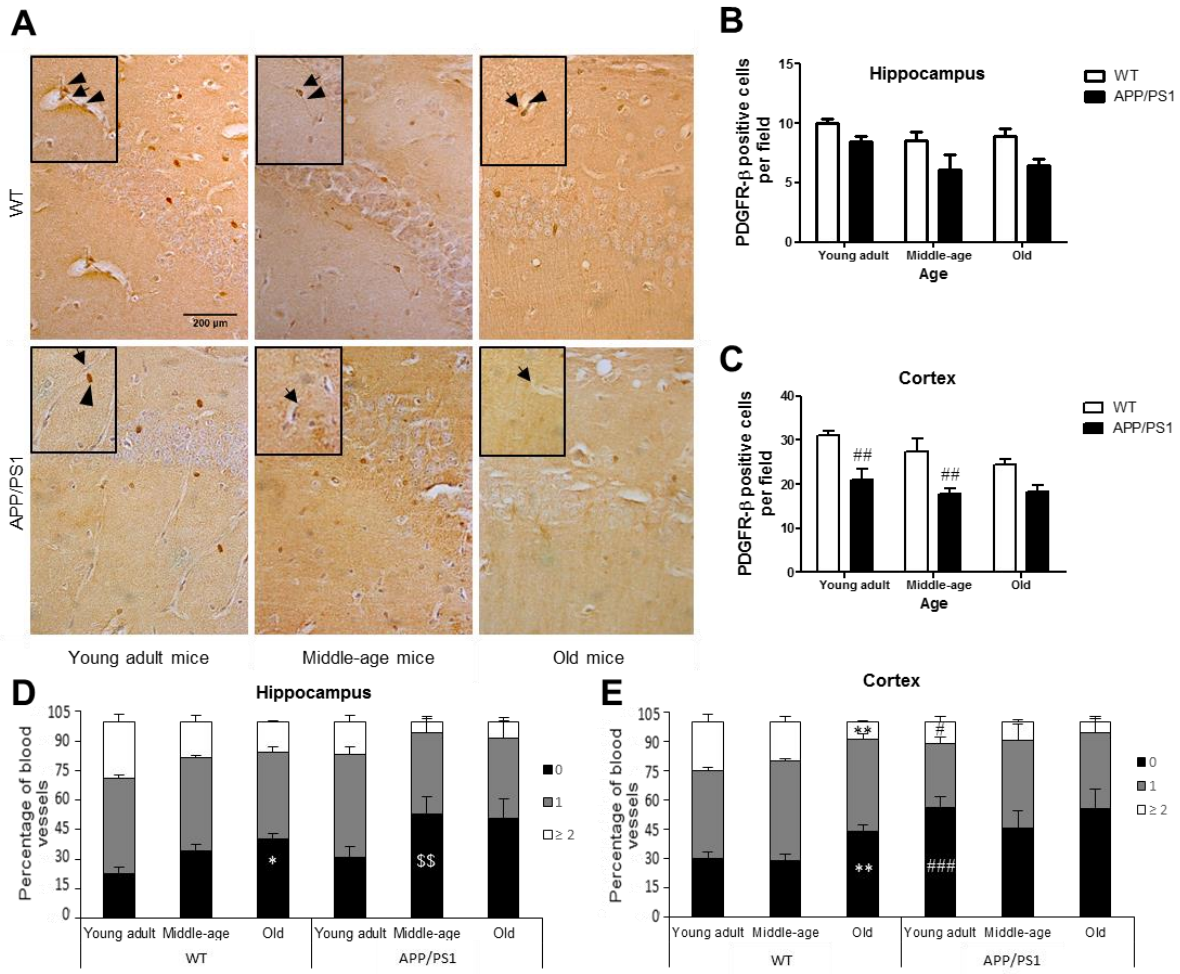

Fig. 3. Changes in the expression of platelet-derived growth factor receptor β (PDGFRβ) by pericytes associated with Alzheimer's disease (AD) and aging in hippocampus and cortex. Brain sections of APP/PS1 transgenic mice, a mouse model of AD, and C57BL/6 wild-type (WT) mice at 6, 12-14 and 23-28 months (young adulthood, middle-age and old, respectively) were processed for immunohistochemical analysis of the pericyte marker PDGFRβ. Representative immunohistological pattern of PDGFRβ in hippocampus (A), where arrows point to capillaries and arrowheads point to PDGFRβ-positive cells, and inserts show PDGFRβ-positive cells around microvessels. Semi-quantitative analysis of PDGFRβ-positive cells per field in hippocampus (B) and cortex (C). $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. age-matched WT. Semi-quantitative analysis of PDGFRβ-positive cells per blood vessel in hippocampus (D) and cortex (E), with results expressed as % of vessels ensheathed by no cell, by 1, or by two or more Iba-1 positive cells. *P<0.05, *P<0.01 vs. WT young adult; $^{\$\$}$P<0.01 vs. APP/PS1 young adult; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.05 vs. WT age-matched.

Interestingly, the thrombin-positive deposits were consistently found at an earlier time point than albumin-positive ones in both genotypes, and the increase of thrombin and albumin positive deposits of in hippocampal parenchyma occurred in an age-dependent manner (two-way ANOVA F 4.28, $P<0.05$ for thrombin), which suggests an increase of BBB permeability with age, independent from AD.

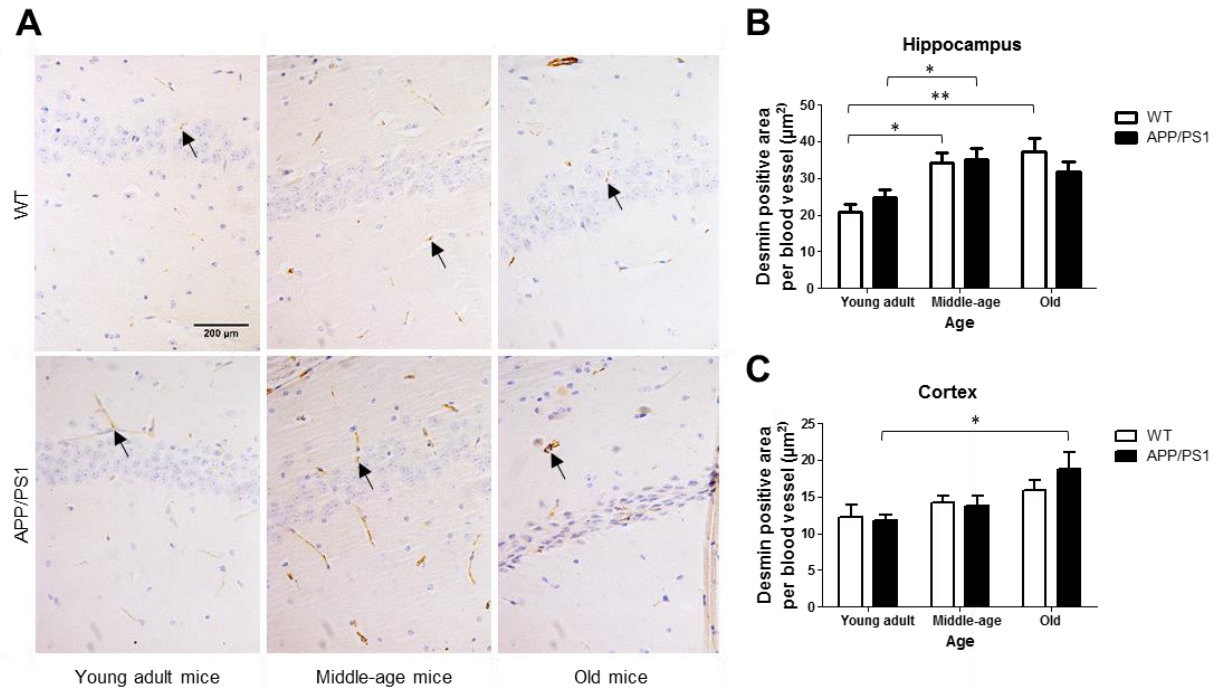

Fig. 4. Changes in the expression of desmin by pericytes associated with Alzheimer's disease (AD) and aging in hippocampus and cortex. Brain sections of APP/PS1 transgenic mice, a mouse model of AD, and C57BL/6 wild-type (WT) mice at 6, 12-14 and 23-28 months (young adulthood, middle-age and old, respectively) were processed for immunohistochemical analysis of the pericyte marker desmin. Representative immunohistological pattern of desmin in hippocampus (A), where arrows point to desmin-positive area. Semi-quantitative analysis of desmin-positive area per blood vessel in hippocampus (B) and cortex (C). $^{*}P<0.05$, $^{**}P<0.01$ vs. between indicated groups.

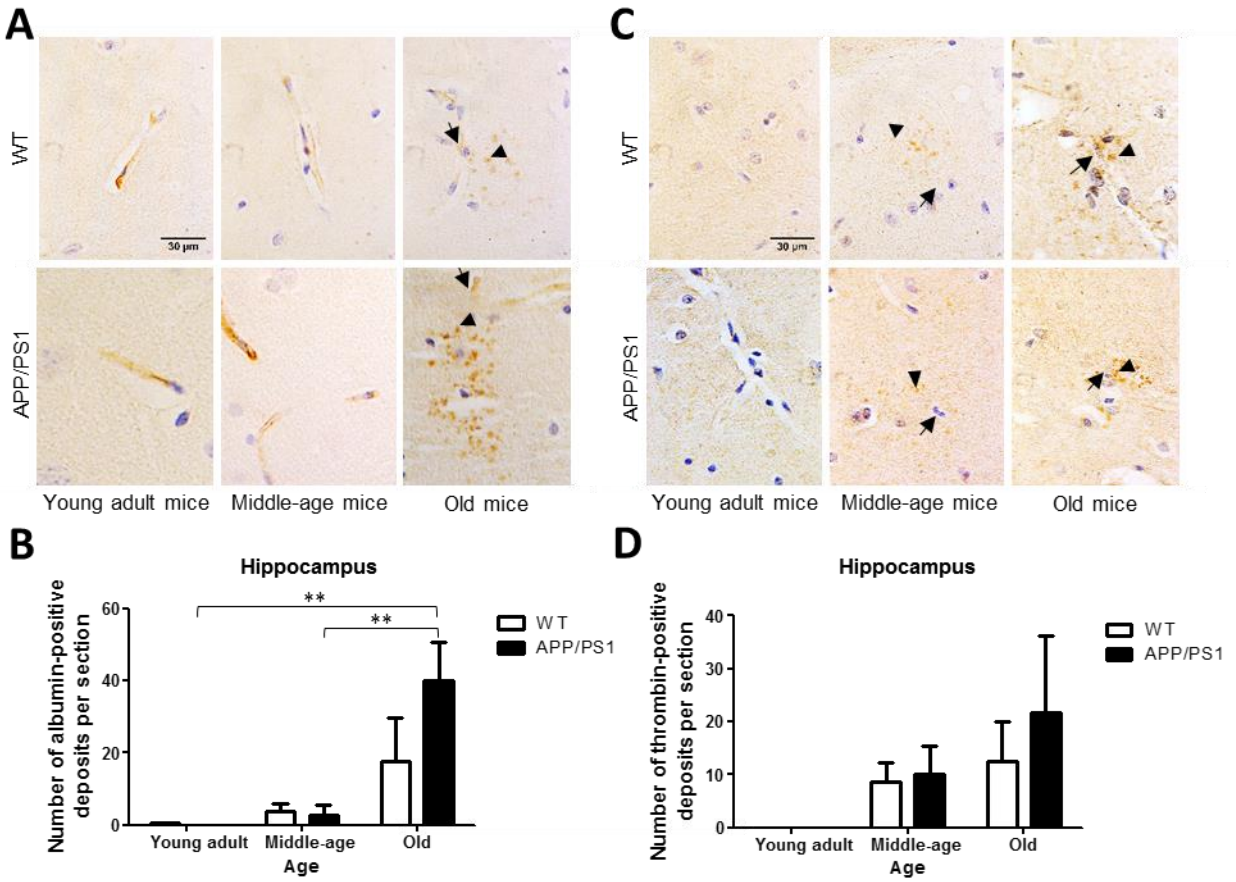

Fig. 5. Entrance of the blood-borne components albumin and thrombin into the hippocampal parenchyma during aging and along Alzheimer's disease (AD) progression. Brain sections of APP/PS1 transgenic mice, a mouse model of AD, and C57BL/6 wild-type (WT) mice at 6, 12-14 and 23-28 months (young adulthood, middle-age and old, respectively) were processed for immunohistochemical analysis of albumin and thrombin. Representative immunohistological pattern of albumin in hippocampus (A) and semi-quantitative analysis of albumin-positive deposits in that region (B). Representative immunohistological pattern of thrombin in hippocampus (C) and semi-quantitative analysis of thrombin-positive deposits in that region (D). The arrows are pointing to capillaries and arrowheads to the positive protein deposits (A and C). $^{**}P<0.01$ vs. between indicated groups.

3.4 Astrocytes and microglia related changes during aging in WT and in AD-like APP/PS1 mice

Previous work suggested that glial activation plays an important role during aging (Jenny, 2012) and that there is an important link between neuroinflammation and AD (Pimplikar, 2014). Hence, we wanted to understand at which time point along healthy aging and disease progression glial activation occurs. To this end, we measured the number of GFAP and Iba-1 positive cells per field and the number of perivascular glial cells (Fig 6,7). Interestingly, the number of GFAP-positive cells in hippocampus of WT mice was much higher than in cortex. Analysis of WT mice showed that while in hippocampus (Fig 6B) the number of GFAP-positive cells did not vary during aging, in cortex (Fig 6C) aging was accompanied by a ~24-fold increase of GFAP-positive cells from young adulthood to old age ($P<0.01$).

Observation of APP/PS1 mice revealed that during disease progression there was an intense rise in astrogliosis in cortex where a ~3-fold increase from young to middle-age was observed ($P<0.001$, Fig 6C), whereas the number of GFAP-positive cells did not vary significantly along time in hippocampus (Fig 6B). Even though not significant, it was interesting to observe a slight decrease from middle-age to old age, suggesting a reduction in astrocyte reactivity and/or density over time. When the WT animals were compared with APP/PS1, APP/PS1 mice had 25% more GFAP-positive cells ($P<0.05$) in hippocampus than WT at middle-age (Fig 6B). The difference between these two groups in cortex was much more robust as APP/PS1 mice had more than ~34-fold more GFAP-positive astrocytes at young adulthood ($P<0.05$), ~14-fold more at middle-age ($P<0.001$) and ~3-fold more ($P<0.001$) at old age than WT mice (Fig 6C). Thus, these results show that the increase of GFAP-positive cells is far more relevant in the cortex than in the hippocampus. Accordingly, the density of GFAP-positive cells in hippocampus (Fig 6B) was affected only by AD (two-way ANOVA F 11.97, $P<0.01$), whereas in cortex (Fig 6C) it was affected equally by aging and AD (two-way ANOVA, F 27.22, $P<0.001$ and F163.5, $P<0.001$, respectively). Furthermore, we observed the perivascular localization of these glial cells by measuring the number of GFAP-positive cells per blood vessel. This measurement was only performed in hippocampus since the low number of these cells in cortex precluded such determination. Analysis of WT and APP/PS1 animals showed that the GFAP-positive cells progressively detached from blood vessels in a very similar way (Fig 6D). From young adulthood to old age, the number of blood vessels with two or more GFAP-positive cells declined from ~20% to ~10% in WT and APP/PS1 mice (Fig 6D).

Regarding Iba-1 quantifications, the number of Iba-1-positive microglia/macrophage cells in WT animals were not changed in hippocampus (Fig 7B), but we observed an increase in cortex of 26% ($P<0.05$) from young adulthood to old age (Fig 7C). In APP/PS1 mice, it was clearly seen that AD was accompanied by microgliosis not only in the cortex, as observed for astrogliosis, but also in hippocampus, where a ~2-fold increase ($P<0.05$ and $P<0.01$, respectively) in the number of Iba-1-positive cells from young adulthood to middle-age was detected in AD-like mice (Fig 7B,C). When WT and APP/PS1 animals were compared we observed that even though at young adulthood both genotypes presented a similar number of Iba-1-positive cells in hippocampus and cortex, by middle-age APP/PS1 mice had already ~2-fold more Iba-1-positive cells than WT animals ($P<0.001$) in both brain regions (Fig 7B, C). This difference was preserved in hippocampus at old age ($P<0.001$), while in the cortex it was less marked (more ~30% than age-matched WT, not significant). Considering the number of Iba-1-positive cells in both regions (Fig 7), we determined that microgliosis was affected by AD (two-way ANOVA F 59.30, $P<0.001$ in hippocampus and F 32.61, $P<0.001$ in cortex) as well as by the interaction of AD and aging of individuals (two-way ANOVA interaction AD × aging F 16.75, $P<0.001$ in hippocampus and F

7.633, P<0.01 in cortex). Regarding the number of Iba-1-positive cells per blood vessel, analysis of the WT animals revealed that during aging, the number of positive cells per blood vessel did not vary in hippocampus and cortex. Analysis of APP/PS1 mice revealed that at the peak of inflammation, there was recruitment of these cells to blood vessels, as indicated by the increase in the number of blood vessels with two or more microglia cells per blood vessel from 10% to 25% and the decrease from 50% to 35% in the number of blood vessels without any Iba-1-positive cells from young adulthood to middle-age in hippocampus (Fig 7D). In sum, considering the glial alterations, it seems that astrocytes react prior to microglia in cortex in APP/PS1 mice and that during aging only cortex presents microgliosis and astrogliosis in WT mice.

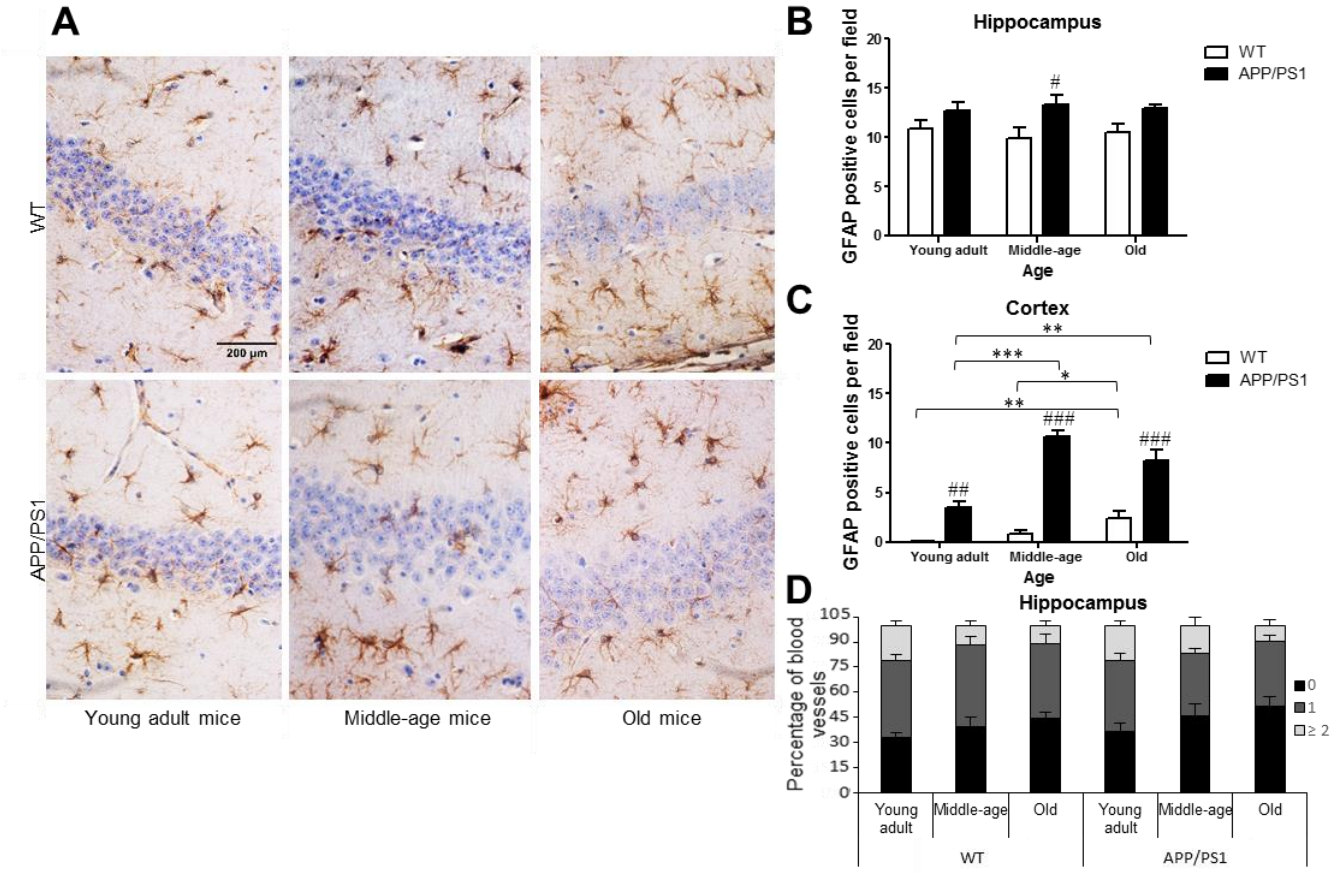

Fig. 6. Astrocyte-related changes associated with Alzheimer's disease (AD) and aging in hippocampus and cortex. Brain sections of APP/PS1 transgenic mice, a mouse model of AD, and C57BL/6 wild-type (WT) mice at 6, 12-14 and 23-28 months (young adulthood, middle-age and old, respectively) were processed for immunohistochemical analysis of the astrocytic marker glial fibrillary acidic protein (GFAP). Representative immunohistological pattern of GFAP in hippocampus (A). Semi-quantitative analysis of GFAP-positive cells in hippocampus (B) and cortex (C). Semi-quantitative analysis of GFAP-positive cells per blood vessel in hippocampus (D), with results expressed as % of vessels ensheathed by no cell, by 1, or by two or more Iba-1 positive cells. **P<0.01, **P<0.01 and ***P< 0.001 vs. indicated groups; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. age-matched WT.

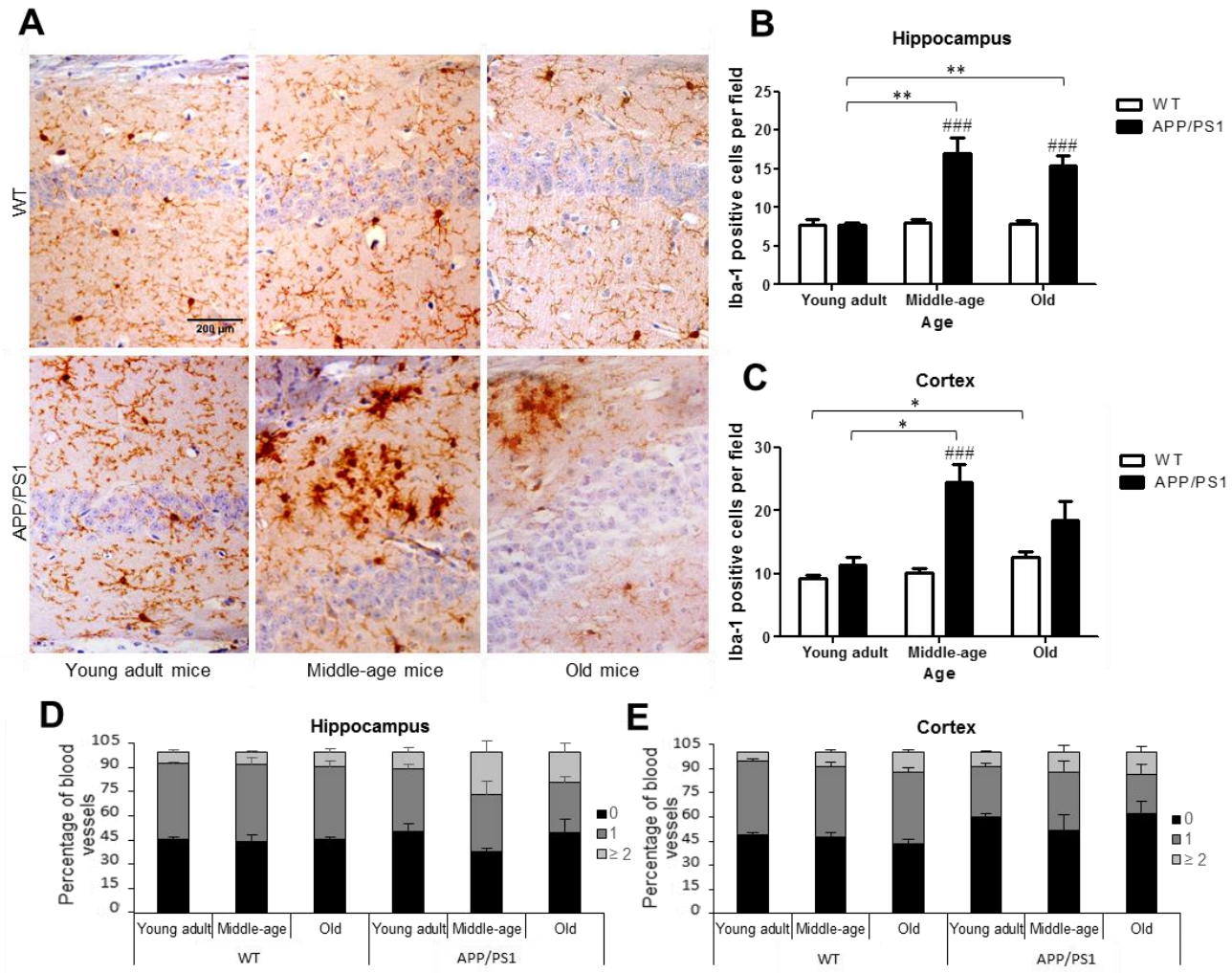

Fig. 7. Microglia related changes associated with Alzheimer's disease (AD) and aging in hippocampus and cortex. Brain sections of APP/PS1 transgenic mice, a mouse model of AD, and C57BL/6 wild-type (WT) mice at 6, 12-14 and 23-28 months (young adulthood, middle-age and old, respectively) were processed for immunohistochemical analysis of the microglial marker ionized calcium-binding adapter molecule 1 (Iba-1). Representative immunohistological pattern of Iba-1 in hippocampus (A). Semi-quantitative analysis of Iba-1-positive cells in hippocampus (B) and cortex (C). Semi-quantitative analysis of vascular coverage by Iba-1-positive cells in hippocampus (D) and cortex (E), with results expressed as % of vessels ensheathed by no cell, by 1, or by two or more Iba-1 positive cells. $^{*}P<0.05$, $^{**}P< 0.01$ vs. indicated groups; $^{\#\#\#}P<0.001$ vs. age-matched WT.

4. Discussion

This study adds to the characterization of AD and aging, by examining vascular density, blood-brain barrier stability and glial activation simultaneously along healthy aging and during disease progression in the hippocampus and cortex of WT and APP/PS1 mouse brain. We conclude that, in both regions, aging resulted in increased RAGE immunoreactivity, desmin expression, as well as in the entrance of thrombin and albumin in hippocampus. On the other hand, AD was the main factor contributing to the loss of PDGFR-β positive cells, in both regions. While hypovascularization was only

a result of aging in cortex, both AD and aging contributed to diminished vascularization in hippocampus. Regarding astrogliosis, whereas in hippocampus it was just an effect of AD, both factors contribute to it in cortex. Lastly, microgliosis was equally affected by aging and AD in both regions.

The frontiers between aging and AD have been greatly debated in the literature, but no consensus yet has been reached. For example, there is no consensus on how aging and AD affect capillary density (Brown and Thore, 2011). However, the majority of studies suggest that a decrease of capillary density in hippocampus and cortex is a hallmark of normal aging and is even more pronounced in AD patients (Bell and Ball, 1986, 1990; Farkas and Luiten, 2001; Fischer et al., 1990), aged healthy mice (Jucker et al., 1990; Sonntag et al., 1997) and AD mouse models (Bailey et al., 2004; Paris et al., 2004), including APP/PS1 mice (Lee et al., 2005), which corroborates our results. However, the factors that lead to the hypovascularization are not entirely understood. Although it was originally hypothesized that an increase in Aβ burden and subsequent BBB disruption were related with the decreased clearance of Aβ from the brain (Shin et al., 2007), an alternative hypothesis states that the vascular dysfunction in AD occurs because amyloidogenesis promotes intense neoangiogenesis contributing to augmented hypervascularization and subsequent vascular permeability (Biron et al., 2011). It is noteworthy to mention that several factors have contributed to the lack of agreement about the effect of aging and AD on cerebral microvasculature (Brown and Thore, 2011), including the use of different animal models, different markers for endothelial cells, differences in brain sections thickness, differences in the caliber of the blood vessel considered and differences in methodologies, especially considering the differences found between 2D and 3D methods. Based on our results, premature hypovascularization occurs in AD, hence, managing and reverting it could be a potential approach to delay the progression of the disease. Moreover, avoiding the loss of vascular density in aged individuals could also be an interesting approach to decrease the odds of developing age-related brain vulnerabilities.

We analyzed how brain microvasculature immunoreactivity of RAGE, a transmembrane receptor responsible for the influx of age glycation endproducts and Aβ, evolved along aging and AD, since most studies focus on the expression of the receptor in whole brain, including neuronal, glial and vascular components. Previous studies showed that normal aged brain is associated with higher expression of RAGE (Cho et al., 2009; Perry et al., 1993), and herein we further show that endothelial cells contribute to this increase. Moreover, AD brains have even higher RAGE expression than aged controls, including in endothelial cells (Jeynes and Provias, 2008; Miller et al., 2008). Accordingly, it was previously demonstrated that APP/PS1 mice (Liu et al., 2014) and other AD mouse model (Cho et al., 2009) have increased RAGE expression in brain homogenates. Contrasting with these reports, here we show that

APP/PS1 mice do not present significant higher RAGE expression than age-matched WT, which raises the hypothesis that in this AD mouse model, RAGE upregulation may occur predominantly in glial cells, where the involvement of this receptor was already demonstrated (Slowik et al., 2012). On the other hand, the previously reported increase of RAGE expression (Miller et al., 2008) was observed in blood vessels with a 5 to 20 μm diameter, whereas the absence of variation here presented was found in capillaries (<6 μm diameter), which suggests that the upregulation occurs in other segments of the vascular tree. When Aβ binds to RAGE, it triggers a signaling pathway that leads to the disruption of tight junctions of BBB (Kook et al., 2012), and activates an intracellular pathway that results in ROS production, mitochondrial dysfunction, and activation of transcription of nuclear factor kappa B (Bierhaus and Nawroth, 2009). Therefore, RAGE upregulation contributes to the development of age-related brain vulnerabilities and triggers several pathways associated with neurodegenerative diseases, rendering this receptor a potential target to modulate (Galasko et al., 2014).

We studied, for the first time, the temporal evolution of pericytes from young adulthood to old age during healthy aging and AD progression using commonly used pericyte markers (Bell et al., 2010; Sá-Pereira et al., 2012). Previous studies reported that there is no pericyte loss during aging in hippocampus and cortex of rodents since there was no change in coverage of capillaries based on analysis of cluster of differentiation 13 up till 9 months old (Sagare et al., 2013b), and of desmin up till 16 months old (Bell et al., 2010). Herein, using transgenic mice with a different genetic background, other pericyte markers, and extending the age of the mice studied to 23-28 months old, we found age-related pericyte alterations, which were already observed at middle-age based on desmin immunostaining. Interestingly, the increased desmin expression in old mice was not accompanied by significant alterations in the total number of PDGFR-β-positive cells but was accompanied by a poorer pericyte vascular coverage. This apparently contradictory finding may result from the fact that pericytes are polymorphic cells with heterogeneous morphology, multiple functional differentiation and migration patterns, and expression of variable markers according to their location in the vascular tree (e.g., pre-, mid- and post-capillaries) (Sá-Pereira et al., 2012). Thus, it is conceivable that PDGFR-β-positive cells may detach from capillaries and the ones that remain in the microvascular wall are those expressing desmin, or even that desmin-positive cells are attracted to the microvasculature. The increase of desmin expression was also reported after ischemic (Yemisci et al., 2009) and traumatic injury (Dore-Duffy et al., 2011), and here, was found along aging in the AD mouse model. Moreover, we found that AD-like transgenic mice have a greater loss of total and perivascular PDGFR-β-positive cells than age-matched controls, which agrees with previous studies (Bell et al., 2010; Sagare et al., 2013b; Sengillo et al., 2013). Previous studies have shown that communication between pericytes and endothelial cells through endothelial platelet derived growth

factor B and pericyte PDGFR-β is vital for pericyte maintenance (Bell et al., 2010). Interestingly, we found that only in cortex, where we detected a very premature and significant detachment of pericytes from blood vessels of APP/PS1 when compared with age-matched WT, there was a decrease in the number of total pericytes. The PDGFR-β pathway is not the only one to maintain pericyte attachment to endothelial cells, but it is crucial for pericyte survival. Thus, the abnormality in this pathway suggested by the reduced expression of PDGFR-β can lead to the detachment of pericytes or recruitment of these cells to other locations (Bonkowski et al., 2011), as well as to their death (Abramsson et al., 2007; Gaengel et al., 2009). On the other hand, pericytes provide important trophic support to blood vessels (Bell et al., 2010; Sá-Pereira et al., 2012). In our study, the loss of PDGFR-β-positive pericytes (observed in young adults) preceded vascular regression, which was observed here as a decrease in blood vessel area detected only at middle-age. Pericytes are also crucial for the maintenance of BBB properties (Sá-Pereira et al., 2012) and its loss contributes to BBB permeability(Bell et al., 2010), as evidenced in our study based on the detection of thrombin and albumin in brain parenchyma, which is in line with previous reports (Armulik et al., 2010; Baloyannis and Baloyannis, 2012; Daneman et al., 2010; Sengillo et al., 2013). Also interesting is the fact that pericyte loss is related to vascular dilatation (Baloyannis and Baloyannis, 2012), as, for example, increased expression of a pericyte protein related to a hypercontractile phenotype, desmin, was observed in the cortex, which also had a significantly fewer PDGFR-β-positive cells and reduced pericyte-vascular coverage. Desmin is a protein with special properties in pericytes, since higher levels of desmin are related with the induction of hypoxia-inducible factor-1α and the upregulation of vascular endothelial growth factor (Shi, 2009), which could be a feedback mechanism to promote angiogenesis, given the age-dependent loss of capillary density (Peppiatt et al., 2006). In recent studies, it was suggested that desmin overexpression in blood-tumor barrier (Lockman et al., 2010), as well as pericyte loss in PDGFR-$\beta^{-/-}$ mice (Bell et al., 2010), lead to BBB disruption, which corroborates our results given the temporal relationship of these events and the increase of thrombin and albumin deposits. Of note in our study, the detection of these two blood-borne components in the parenchyma over time does not happen in the same magnitude or at the same time point, since thrombin was detected prior to albumin and the number of albumin deposits was twice the number of thrombin deposits. This could be due to the fact that albumin has a higher molecular weight than thrombin (67 and 37 kD, respectively), which means that a higher magnitude of BBB disruption would be required for albumin leakage into brain parenchyma as compared to the impairment that allows thrombin passage. However, we found a higher number of albumin deposits in brain parenchyma than thrombin, which may simply be due to the fact that albumin is the most common serum albumin. The

increase of these proteins in the brain parenchyma was a later event in our mice, suggesting that BBB disruption occurs as a result of endothelial and pericyte changes, as observed by others (Bell et al., 2010).

Our results are in agreement with previous reports showing that astrogliosis and microgliosis are both detected in frontal cortex of APP/PS1 mice by 15 months of age (Kamphuis et al., 2012; Wirz et al., 2013). Similar to a previous study, we detected increased microgliosis but not astrogliosis in the hippocampus of APP/PS1 mice (Wang et al., 2010b). This is interesting because it contradicts the hypothesis that microgliosis could contribute in a very early phase of the disease to pathogenesis, at least in this model. It is also noteworthy to mention that at the peak of inflammation in APP/PS1 mice, when microglia are activated, these cells migrate towards blood vessels, as was made evident by the fact that the number of perivascular microglia increases. Moreover, given that neuroinflammation is a feature of AD, it is likely that this microglia recruitment is related to the immune response at the BBB. Accordingly, a recent study reported that neuroinflammation contributes to the increase of BBB permeability in an AD mouse model (Takeda et al., 2013). Thus, it is possible that the increase in perivascular microglia contributes to BBB dysfunction (Nishioku et al., 2010) instead of contributing to restore the BBB integrity, as was suggested by Willis and colleagues (Willis, 2011). Remarkably, BBB dysfunction is also observed in other neurodegenerative diseases, such as Parkinson's disease (Bartels, 2011), multiple sclerosis (Tourdias and Dousset, 2013) and amyotrophic lateral sclerosis (Winkler et al., 2014), thus, the role of neuroinflammation in BBB stability warrants further study. Also, we observed a slight increase of perivascular microglia in cortex but not hippocampus in the aged brain of old WT mice compared with young adult WT mice. Astrocytes behave in the opposite way, since aging of both WT and APP/PS1 mice is accompanied by a decrease of perivascular astrocytes in hippocampus compared with young adult genotype-matched animals.

Lastly, we established for the first time that while APP/PS1 mice already begin to deposit senile plaques by young adulthood in hippocampus and cortex, these regions are not affected by the same vascular and glial events simultaneously. This may indicate that these changes are not dependent upon or related to the accumulation of Aβ, as the burden of Aβ seems to be similar in these two regions. Our findings suggest that at young adulthood, Aβ accumulation in hippocampus precedes vascular and glial changes. In contrast, Aβ accumulation in cortex occurs concurrently with a reduction in PDGFR-β-positive cells and astrogliosis. In figure 8 the temporal evolution of glial and vascular events, along with the appearance of senile plaques is schematically represented.

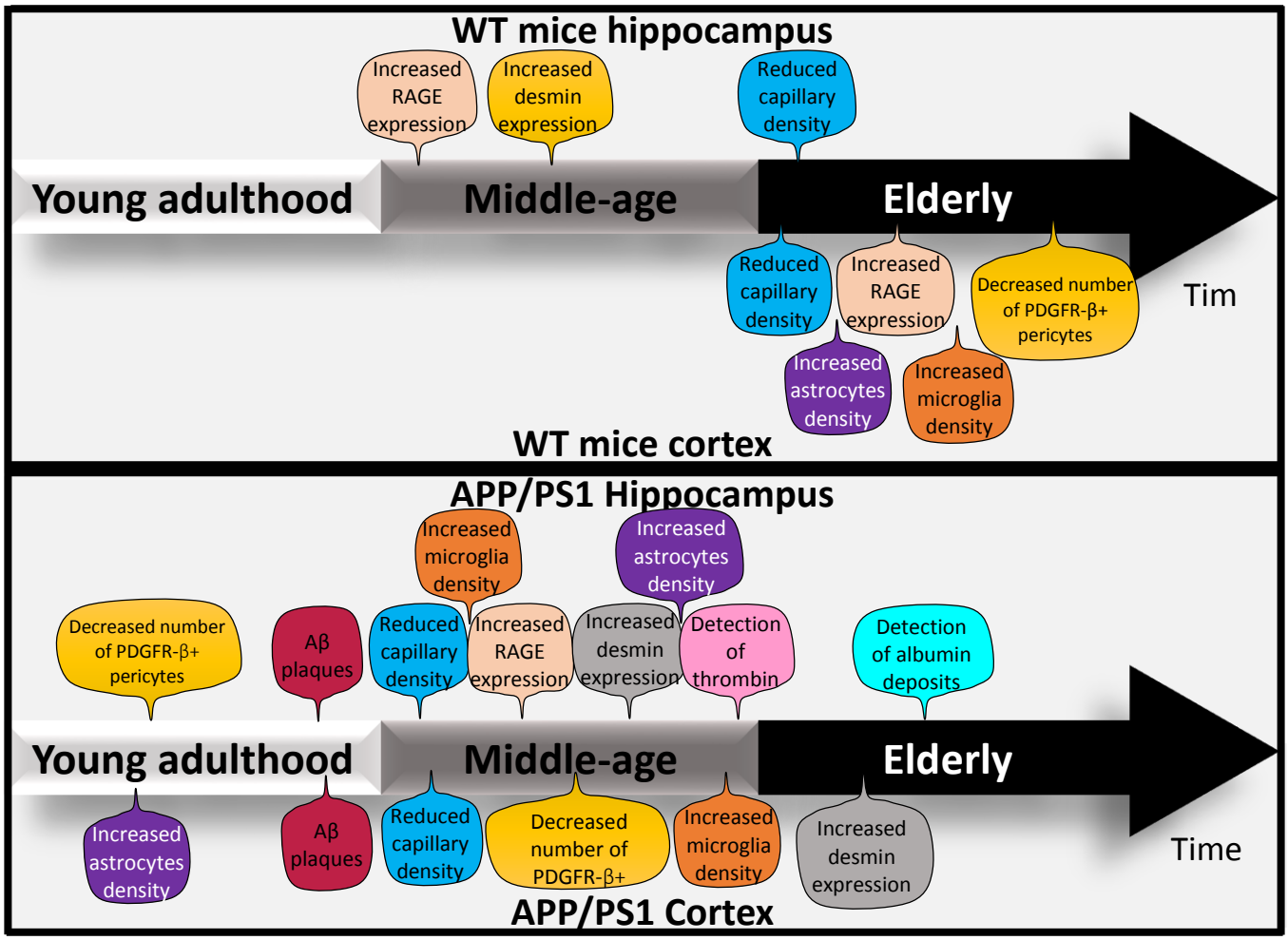

Fig. 8.The temporal evolution of vascular and glial changes, along with the appearance of senile plaques in cortex and hippocampus of APP/PS1 transgenic mice, a mouse model of Alzheimer's disease, and C57BL/6 wild-type (WT) mice.

In sum, our findings suggest that it would be interesting to start targeting vascular and glial alterations in the elderly, as such alterations seem to predispose the elderly to age-related brain vulnerabilities. For example, it is likely that BBB stabilizing drugs or drugs that contribute to vascular density maintenance may have beneficial effects in elderly. Our results strongly suggest that AD pathogenesis, e.g. Aβ deposition in hippocampus, further accentuates vascular and glial changes, at least in mice, which confirms that vascular and glial alterations play an important role in AD pathogenesis. This may justify further studies to evaluate the benefits of vasculature modifying drugs not only in sporadic AD, but also in familial AD patients, because even though there may be differences in the individual pathological cascades, it is clear that vascular changes contribute to disease progression.

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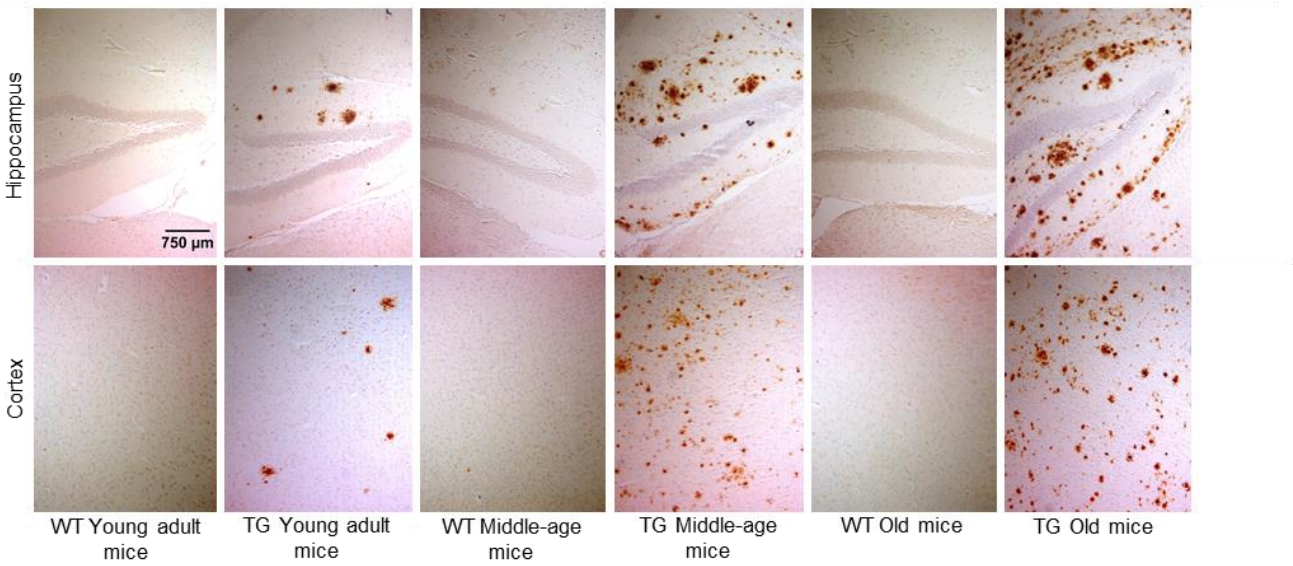
Supplemental figures



Supplemental figure 1. The temporal evolution of amyloid-β (Aβ) burden in the hippocampus and cortex of C57BL/6 wild-type (WT) and APP/PS1 transgenic (APP/PS1), a mouse model of AD. Brain sections of APP/PS1 transgenic mice, a mouse model of AD, and C57BL/6 wild-type (WT) mice with 6 months old were processed for immunohistochemical analysis of the Aβ (produced by Selkoe Lab) and the density of senile plaques was analyzed semi-qualitatively. Representative immunohistological pattern of amyloid- β in the hippocampus and cortex.

Chapter III

Final considerations

Conclusions and perspectives

What we know about BBB today is a result of a very fruitful decade, along which a lot of studies identified new mechanistic insights within BBB maintenance. Moreover, it was found that miscommunication between neurovascular unit cells, as well as disturbances in the homeostasis of each cell, could have a major impact in CNS functioning. Today, BBB dysfunction is recognized as an important player in the pathogenesis of several neurodegenerative diseases, such as Alzheimer's disease, multiple sclerosis and amyotrophic lateral sclerosis. Nevertheless it is still unclear if it is an upstream or downstream event in sporadic AD. Interestingly, sealing the BBB in patients with neurodegenerative diseases may be a very effective approach in slowing down the disease progression. Afterwards, it is important to understand if not only BBB disruption, but all the BBB dysfunction (including transporters dysfunction and abnormal protein expression by BBB cells) and is a reversible event. Since BBB has intrinsic skills to repair itself, it could be interesting to find out which are most suitable and easier to modulate therapeutically. In the last years, we gained a new perspective about the importance of TJ proteins for BBB stability. Therefore, the development of a therapeutic approach that could upregulate the expression of TJ proteins could be a modulatory therapy of AD progression. For the evaluation of these approaches and to better understand its impact in the disease pathogenesis, it is also needed to develop better, and more complex BBB models that include NVU cells, as well as disease models that could better mimic all the hallmarks of each disease, which will take several years to achieve. In fact, it is important to note that we used a mice model that mimics the familial early onset of AD, thus, it would be very interesting to perform this kind of study that aims to study the vascular dysfunction in AD pathogenesis using a mice model that harbors a risk factor. For examples, it would be interesting to perform a similar study using the mice model that carries the 4 allele of ApoE, which is a major risk factor for sporadic AD, as well as use an AD mice model of sporadic AD, such as streptozocin (icv-STZ) mouse an create an environment that will put the animal under stress, increase risk factors as high blood pressure, or to include a lipidic diet in mice quotidian. This approach would be exciting since it would help us to better understand if and how vascular parameters contribute to the pathogenesis of sporadic AD. In this way, if they play a major role in the pathogenesis, they could be considered as therapeutical targets and it would be possible to reduce the odds to aged brain to develop the disease. Moreover, it could be interesting to investigate if, in earlier time points, in this mice model we could observe BBB damage before neuronal impairment.

Despite the huge progress in the current knowledge about the mechanisms behind neurodegeneration, important questions remain unanswered. Therefore, further efforts should be

envisaged in order to clarify how aging predisposes to neurodegenerative diseases in general and to AD in particular, to decipher the contribution of the different players in the orchestra of neurodegeneration, and to disclose the contribution of vascular dysfunction in the pathogenesis of the disease. Hopefully, clarification of these issues will reveal reliable biomarkers to predict individuals at risk, essential to timely establish preventive attitudes to avoid disease occurrence or, at least, delay the disease progression. Moreover, a better comprehension of cellular alterations and interplay within the neurovascular unit, as well as signaling pathways at a cellular level, is pivotal for the identification of novel targets to modulate the disease burden.